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ADHERENCE OF BACTERIA TO HYDROCARBONS: A SIMPLE METHOD FOR MEASURING CELL-SURFACE HYDROPHOBICITY

M. ROSENBERG, D. GUTNICK and E. ROSENBERG

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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1. Introduction

The hydrophobic nature of the outermost surface of various microbial cells has been implicated in such biological phenomena as interactions between bacteria and phagocytes [1], attachment of bacteria to host tissue [2,3], adherence of bacteria to non-wettable solid surfaces [4,5], partitioning of bacteria at liquid : liquid [6] and liquid : air [7,8] interfaces, and the ability of microbial cells to grow on hydrocarbons through direct contact with the immiscible substrate [9–12].

A number of methods for studying hydrophobic interactions of cells have been reported in the literature. These include binding of hydrocarbon and fatty acids to cells and cell components [12,13], measurement of the force required to remove hydrocarbon-bound cells [9], partitioning of bacteria in aqueous polymer two-phase systems [14,15], hydrophobic interaction chromatography [3,15] and contact angle measurements of dried cell layers [1,16]. No single method adequately describes cell-surface hydrophobicity since (1) the experimental conditions employed influence the observed hydrophobic interactions to some degree and (2) various non-hydrophobic effects often interfere.

Interest in the mechanism which enables direct contact between hydrocarbon-degrading cells and their water-insoluble alkane substrates has led us to develop a rapid quantitative assay for the hydrophobic interaction of cells with liquid hydrocarbons. This method is based on the degree of adherence of cells to various liquid hydrocarbons following a brief period of mixing. The present report describes this technique and its application in measuring the surface hydrophobicity of various bacterial cells.

2. Materials and Methods

2.1. Bacteria

The following test bacteria were used: *Acinetobacter calcoaceticus* strain RAG-1 (ATCC 31012) was isolated previously in this laboratory [17,18]; *A. calcoaceticus* strain BD 413 *tryp* E 27 was kindly provided by Dr. E. Juni. *Escherichia coli* B *ilvA thy*, *E. coli* K-12 CSH 57, *E. coli* J-5, *Bacillus subtilis* 168 and *Enterobacter aerogenes* CDC 659/66 were kindly provided by Dr. E.Z. Ron. *Micrococcus lysodeikticus* ATCC 4698 was kindly provided by Dr. I. Friedberg. Locally isolated strains of *Staphylococcus aureus*, *Staphylococcus albus* and *Serratia marcescens* were kindly provided by Ruth Zack. *Pseudomonas aeruginosa* PAS 279 was kindly provided by Dr. J. Shapiro. The test bacteria were grown at 30°C with shaking in nutrient broth. Unless otherwise stated, bacteria were harvested at early logarithmic growth phase, washed twice and resuspended in PUM buffer, pH 7.1: 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml.

2.2. Assay procedure

To round-bottom test tubes (10 mm diameter), containing 1.2 ml of washed cells suspended in PUM buffer, were added various volumes of test hydrocarbon (n-hexadecane, n-octane or p-xylene). Following 10 min preincubation at 30°C, the mixtures were agitated uniformly on a Thermolyne Maxi Mix (Sybron) for 120 s. After allowing 15 min for the hydrocarbon phase to rise completely, the aqueous phase was carefully removed with a Pasteur pipette and transferred to a 1 ml cuvette. Light absorbance was determined at 400 nm, using a Gilford Model 240 spectrophotometer.

3. Results

The basic experiment used to measure bacterial hydrophobicity is shown qualitatively in Fig. 1. Hexadecane was layered onto a turbid aqueous suspension of *Acinetobacter* RAG-1 (Fig. 1, left tube) and then mixed for 120 s; on standing a clear bottom layer and a "creamy" upper layer formed (Fig. 1, middle tube). Microscopic examination of the upper layer revealed an oil-in-water emulsion consisting of hexadecane droplets covered with patches of bacteria (Fig. 2). Decrease in absorbance of the lower aqueous phase was used as a measure of cell surface hydrophobicity. The cell-stabilized emulsions did not break even after several days; however, addition of 5% (v/v) isopropanol brought about an immediate coalescence of droplets and release of cells into the aqueous phase (Fig. 1, right tube).

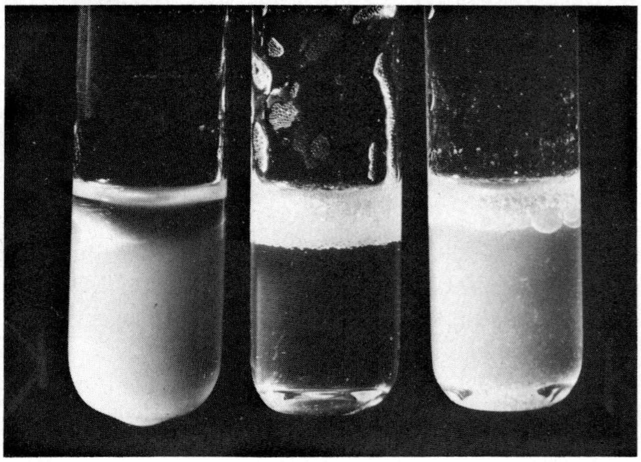

Fig. 1. Adherence of *Acinetobacter* RAG-1 to hydrocarbon. Hexadecane was added to an aqueous suspension of *Acinetobacter* RAG-1 (left). After mixing for 120 s and allowing to stand, adherent cells rose with the hydrocarbon, forming a "creamy" upper layer and a clear aqueous phase (middle). Addition of isopropanol to a final concentration of 5% (v/v) results in breakage of the emulsion and release of cells back into the aqueous phase (right).

Results illustrating the adherence of various bacteria to the test hydrocarbons are presented in Figs. 3–6. Fig. 3 compares the affinity of *E. coli* with those of two *Acinetobacter* strains. *E. coli* B showed no significant affinity towards hexadecane or octane and little or no affinity towards xylene. Similar results (not presented) were obtained with *M. lysodeikticus, E. aerogenes, B. subtilis* and *P. aeruginosa.* While *Acinetobacter* RAG-1 showed high affinity towards all three hydrocarbons, *Acinetobacter* BD exhibited higher affinity for octane and xylene than for hexadecane. More than 97% of the RAG-1 and over 99% of the BD strain cells were removed from the aqueous phase by 0.2 ml octane.

The affinities of two species of *Staphylococci* towards the test hydrocarbons are presented in Fig. 4. Although *S. albus* did not adhere to hexadecane or xylene, *S. aureus* exhibited a high affinity towards all the test hydrocarbons.

Although *S. marcescens* grown to early exponential phase showed relatively low affinity towards hexadecane and xylene and none towards octane, pink-pigmented early stationary phase cells adhered strongly to all three hydrocarbons (Fig. 5).

Loss of oligosaccharide components on the outer surface of *E. coli* rough mutant J-5 [19] was accompanied by a significant increase in its affinity towards

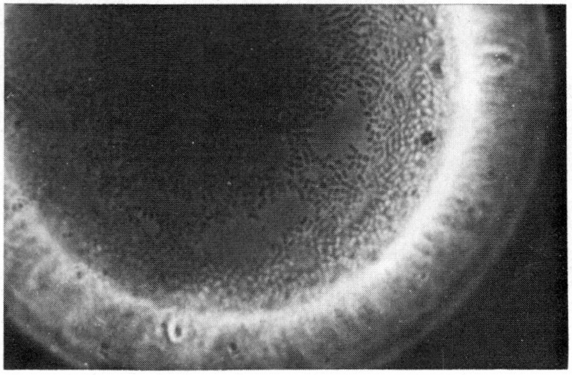

Fig. 2. Phase micrograph showing **RAG-1** cells adhering to hexadecane droplet following mixing. 825×.

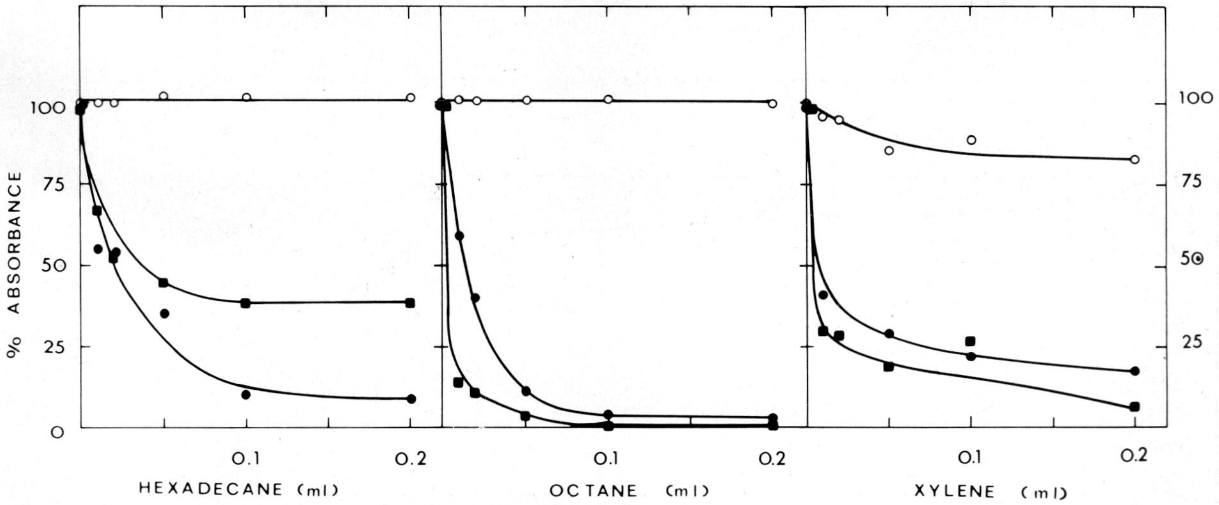

Fig. 3. ○, *E. coli* B (1.562); ●, *Acinetobacter* RAG-1 (1.441); ■, *Acinetobacter* BD (0.632).

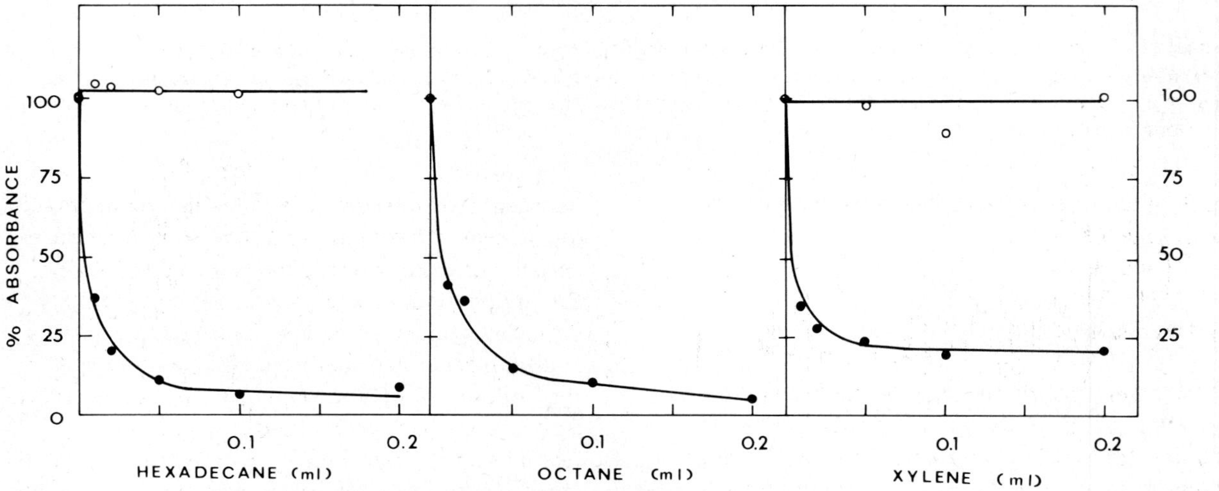

Fig. 4. ○, *Staphylococcus albus* (0.716); ●, *Staphylococcus aureus* (1.175).

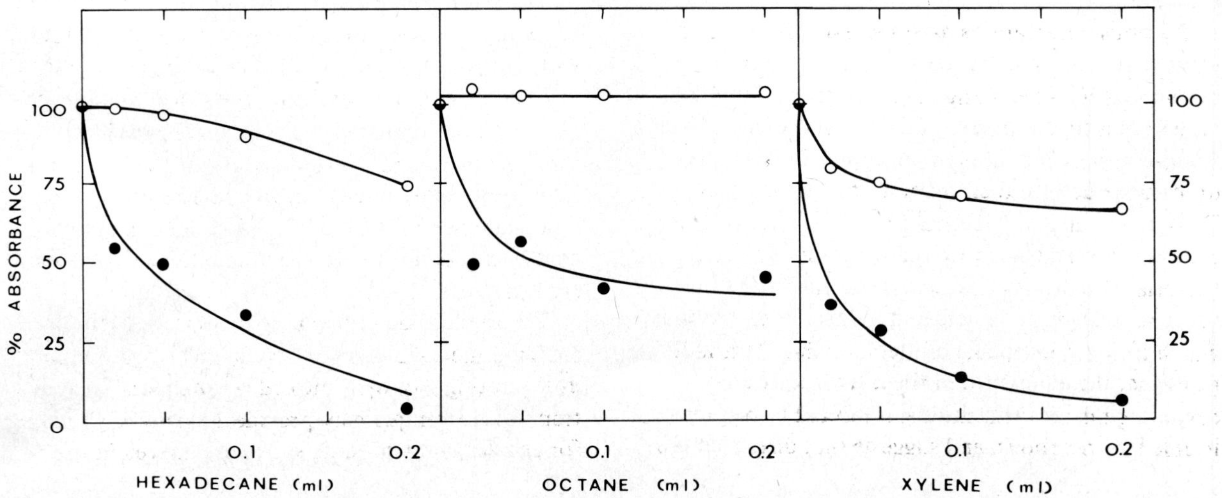

Fig. 5. ○, *Serratia marcescens*, early logarithmic phase (1.120); ●, *Serratia marcescens*, early stationary phase (1.132).

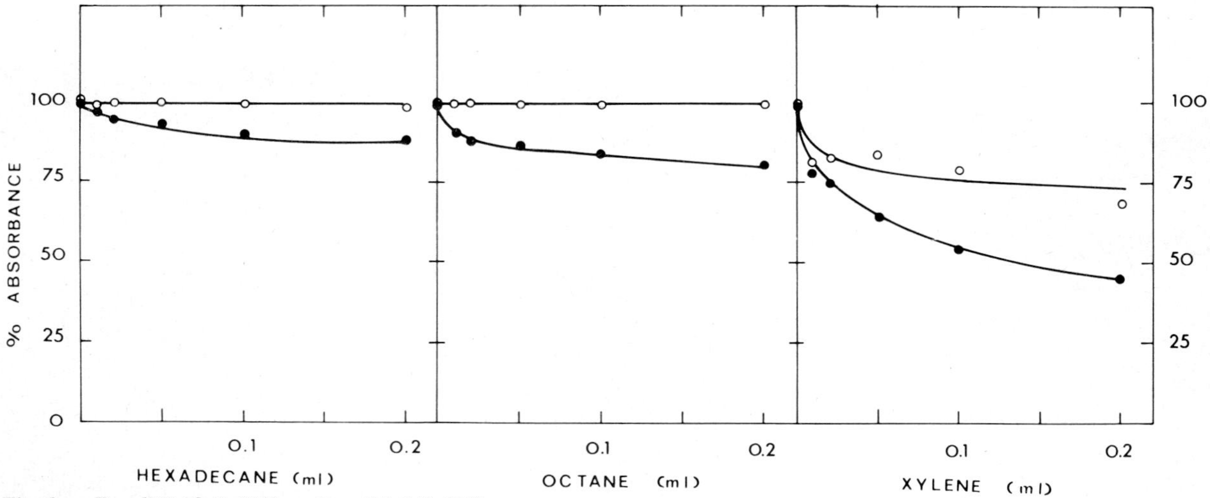

Fig. 6. ○, *E. coli* K-12 (1.558); ●, *E. coli* J-5 (1.408).

Figs. 3–6. Affinity of bacteria towards hydrocarbon as a function of hydrocarbon volume. Aqueous bacterial suspensions were mixed with varying volumes of hexadecane, octane and xylene as described in Materials and Methods. Results are expressed as percentage of the initial absorbance (*A* 400) of the aqueous suspension as a function of hydrocarbon volume. Initial absorbance is shown in parentheses.

each of the three test hydrocarbons, as compared with *E. coli* K-12 (Fig. 6).

4. Discussion

A simple quantitative method has been described for studying the outer cell surface of bacteria, based on the affinity of these cells for liquid hydrocarbons. Large differences in the affinities of various bacteria for hydrocarbons have been demonstrated using this technique.

Previous observations have suggested that the ability to adhere to bulk hydrocarbon is a characteristic of hydrocarbon-degrading microorganisms [11]. We have shown that *S. aureus* and early stationary phase *S. marcescens* cells adhere to a variety of liquid hydrocarbons despite their inability to degrade them. Thus, the ability of bacterial cells to adhere to hydrocarbon is not restricted to hydrocarbon-degrading bacteria. Moreover, both *Acinetobacter* strains adhered strongly to octane and xylene, hydrocarbons which they are incapable of metabolizing. This indicates that the adherence of these hydrocarbon-degrading microorganisms is not limited to metabolizable hydrocarbons, and suggests that direct contact between *Acinetobacter* cells and bulk hydrocarbon is due to general hydrophobic interactions rather than specific recognition of the substrate. *P. aeruginosa* PAS 279 did not adhere significantly to the test hydrocarbons under the conditions studied, despite its ability to grow on hexadecane. This result suggests that affinity for hydrocarbon may vary among hydrocarbon-degrading bacteria. Further studies on the relationship between adherence to hydrocarbon and growth of hydrocarbon-degrading bacteria are underway.

The large increase in hydrophobicity observed in *S. marcescens* with increasing age of the cells agrees with previous reports [8,13]. The small, but significant increase in the affinity of the rough mutant *E. coli* J-5 as compared with that of *E. coli* K-12 indicates the increased hydrophobicity of the rough mutant, presumably due to the increased exposure of inner core regions of the LPS layer. Similar results have been obtained with rough mutants of *Salmonella typhimurium* [1,14].

The method described here may prove useful in enabling the separation of certain cell mixtures, and in separating hydrophobic cell components. In addition, this technique may provide a means of enriching for and selecting mutants which are altered in their surface hydrophobicity. A search for such mutants is currently in progress, with a view towards investigating the relationship between the metabolism of hydrophobic substrates and modifications in cell surface hydrophobicity.

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References

[1] Cunningham, R.K., Söderström, T.O., Gillman, C.F. and Van Oss, C.J. (1975) Immunol. Commun. 4, 429–442.

[2] Perers, L., Andäker, L., Edebo, L., Stendahl, O. and Tagesson, C. (1977) Acta Pathol. Microbiol. Scand. Sect. B 85, 308–316.

[3] Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, T. (1978) Infect. Immun. 22, 462–472.

[4] Dexter, S.C., Sullivan, J.D., Jr., Williams, J., III and Watson, S.W. (1975) J. Gen. Microbiol. 30, 298–308.

[5] Fletcher, M. and Loeb, G.I. (1979) Appl. Environ. Microbiol. 37, 67–72.

[6] Marshall, K.C. and Cruickshank, R.H. (1973) Arch. Mikrobiol. 91, 29–40.

[7] Stanley, S.O. and Rose, A.H. (1967) J. Gen. Microbiol. 48, 9–23.

[8] Blanchard, D.C. and Syzdek, L.D. (1978) Limnol. Oceanogr. 23, 389–400.

[9] Miura, Y., Okazaki, M., Hamada, S.-I., Murakawa, S.-I and Yugen, R. (1977) Biotech. Bioeng. 19, 701–714.

[10] McLee, A.G. and Davies, S.L. (1972) Can. J. Microbiol. 18, 315–319.

[11] Kennedy, R.S., Finnerty, W.R., Sudarsanan, K. and Young, R.A. (1975) Arch. Microbiol. 102, 75–83.

[12] Käppeli, O. and Fiechter, A. (1976) Biotech. Bioeng. 18, 967–974.

[13] Kjelleberg, S., Lagercrantz, C. and Larsson, Th. (1980) FEMS Microbiol. Lett. 7, 41–44.

[14] Magnusson, K.-E. and Johansson, G. (1977) FEMS Microbiol. Lett. 2, 225–228.

[15] Magnusson, K.-E., Stendahl, O., Stjernström, I. and Edebo, L. (1978) Acta Pathol. Microbiol. Scand. Sect. B 86, 113–120.

[17] Reisfeld, A., Rosenberg, E. and Gutnick, D. (1972) Appl. Microbiol. 24, 363–368.

[18] Horowitz, A., Gutnick, D. and Rosenberg, E. (1975) Appl. Microbiol. 30, 10–19.

[19] Braude, A.I. and Douglas, H. (1972) J. Immunol. 108, 505–512.

Adherence of Bacteria to Hydrocarbons

E. Rosenberg, M. Rosenberg, and D. L. Gutnick
Department of Microbiology
George S. Wise Faculty of Life Sciences
Tel Aviv University
Ramat Aviv, Israel

ABSTRACT

Two general types of interaction between microorganisms and hydrocarbons have been postulated: (A) adherence of cells directly to large oil droplets and (B) release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon:aqueous interfacial area. In the case of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 both types of interaction are operative; cells adhere avidly to test hydrocarbons (xylene, octane, hexadecane and crude oil) and also produce a potent polyanionic emulsifier referred to as emulsan. Mutants of *A. calcoaceticus* RAG-1 deficient in emulsan synthesis are still able to adhere to hydrocarbons and grow on hexadecane or crude oil as the sole source of carbon and energy. However, mutants of *A. calcoaceticus* RAG-1 unable to adhere to hydrocarbons also failed to grow on hydrocarbon substrates.

It was demonstrated that adherence is a prerequisite for growth on hexadecane under two conditions: low initial cell density and limited emulsification of the substrate. Such conditions prevail in most natural environments. On the other hand, bioemulsification is a cell density dependent phenomenon. Relatively high cell densities are required to produce enough extracellular emulsifying agent to markedly affect the hydrocarbon substrate.

Adherence of microorganisms to hydrocarbons is neither an exclusive property of hydrocarbon-degrading microorganisms nor restricted to those hydrocarbons that the microorganism can metabolize. For example, *Staphylococcus aureus, Serratia marcescens* and *Streptococcus pyogenes* adhered avidly to test hydrocarbons as a result of their high cell surface hydrophobicity, but were unable to metabolize any of the hydrocarbon substrates tested. *A. calcoaceticus* RAG-1 can grow on alkanes, but not aromatics; however, it adhered equally well to both substances. Further, certain bacteria that have the genetic potential to degrade hydrocarbons, e.g., *Pseudomonas aeruginosa,* adhere poorly to hydrocarbons. It follows that introduction of hydrocarbon-degrading plasmids into microorganisms with low cell surface hydrophobicity may not lead to cells that interact well with hydrocarbons in open systems.

INTRODUCTION

The importance of cell adherence in growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces.[36] The initial step in the life cycle of *Bdellovibrio* is attachment to Gram-negative bacteria.[39,41] Specific cell-to-cell interactions pervade the entire life cycle of the myxobacteria.[8,38] Adhesion is a prerequisite to the successful colonization by microorganisms of animals[24] and plants.[18] Microbial adhesion to surfaces has been the subject of several recent reviews.[6,17,18,19,24]

In cases where the carbon or energy source is a water-insoluble material, such as cellulose, or chitin, bacterial adhesion can facilitate growth,[17,38,40] but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, growth of microorganisms on hydrocarbons presents a special problem since not only are hydrocarbons immiscible with water, but also their breakdown cannot occur extracellularly. The first step in aromatic[12] or aliphatic[27] hydrocarbon degradation is introduction of molecular oxygen into the molecules by cell-associated enzymes.[10]

Two general types of hydrocarbon-cell interactions have been postulated, depending upon the state and size of the oil droplets relative to the size of the microbial cells:[15] adherence of cells to large oil droplets and pseudosolublization involving the cellular assimilation of emulsified small hydrocarbon droplets. The relative contribution of these two types of interactions to the growth of bacteria on hydrocarbon has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon: aqueous interfacial area and growth rate can be used to support either hypothesis.[22]

Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which bacterial hydrophobicity and adherence to hydrophobic surfaces are involved. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in the partitioning of microorganisms at the air:water interface,[4,19] in adherence of bacteria to nonwettable plastic surfaces,[7,9,11,31] and in the attachment of bacteria to phagocytes[42] and other mammalian cells.[2,25,33,37]

We previously described a rapid and quantitative method for the measurement of bacterial adherence to hydrocarbons.[32,34] The present report deals with the use of this technique in (1) examining the cell surface hydrophobicity of various bacterial species, some of which do not metabolize hydrocarbons, and (2) selecting and isolating non-adherent mutants. The isolation of a non-adherent mutant of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 has enabled us to examine the role of adherence in bacterial growth on hexadecane.

MATERIALS AND METHODS

Bacterial strains—Tables 1 and 2 contain a list of bacteria used in this study. *Acinetobacter calcoaceticus* MR-481 is a non-adhering derivative of strain RAG-1.

Media and growth conditions—For measuring adherence to test hydrocarbons or preparing inocula for growth experiments, 0.1 ml bacteria of an overnight culture were inoculated into 50 ml growth medium and incubated in a New Brunswick Model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested at the end of exponential phase, washed twice and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_3O$; 7.26 g KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$, and distilled water to 1000 ml, pH 7.1). Growth media were: BHI—brain heart infusion broth (Difco); hexadecane medium—PUM buffer supplemented with 0.2 percent (v/v) hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland). P-hexadecane medium—5 g NH_4NO_3 , 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 ml hexadecane, 50 mg threonine and tap-water to 1000 ml; NB—nutrient broth (Difco) supplemented with 0.5 percent NaCl. CT medium contained 1 percent casitone (Difco) and 0.2 percent $MgSO_4 \cdot 7H_2O$.[29]

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14 mm diameter) containing 4.2 ml aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2 percent sodium acetate. Hexadecane growth medium consisted of 0.5 ml hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick gyrotory water bath shaker (Model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Adherence to hydrocarbons—The technique for measuring adherence of bacteria to hydrocarbons has been described previously.[24] Cells were washed twice and resuspended in PUM buffer to an initial A_{400} of approximately 1.5. Various volumes of the test hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed cell suspension and the phases were vortexed uniformly for 120 sec. After allowing 15 min for the phases to separate, the absorbence of the lower aqueous phase of each tube was measured at 400 nm (Gilford model 240 spectrophotometer, 1 cm light path). Adherence was calculated as the percentage loss in absorbence relative to that of the initial cell suspension.

Isolation of the non-adherent mutant MR-481—A spontaneous mutant of RAG-1 which is deficient in its ability to adhere to hydrocarbons was isolated by the following technique: RAG-1 cells were inoculated into 50 ml nutrient broth medium (NB) and incubated in a New Brunswick model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested in stationary phase following 18 hr growth, washed twice and resuspended in PUM buffer to an A_{400} of approximately 1.5. To 5 ml of the cell suspension were added 2 ml of n-octane, and the phases vortexed for 5 min. Following phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases were mixed as described previously. This extraction process was continued until cells in the lower aqueous phase were too few to be visible under microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to stationary phase as previously described. Cells were again harvested, washed and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. The final aqueous phase was plated on nutrient agar, and three individual colony types were isolated and tested for adherence to hydrocarbons. One clone of cells, termed MR-481, exhibited no significant affinity towards the test hydrocarbons, and was chosen for further study.

Emulsan preparation—The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously.[30] The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH Chem. Ltd.), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing extensively against distilled water. The material was then deproteinized by the phenol method[45] and dialyzed. The final product had a reduced viscosity of 490 cc per g, an emulsifying activity of 150 units per mg, an ester content of 0.5 μmoles per mg and contained less than 0.5 percent protein. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and sterilized by autoclave prior to its addition in the growth experiments.

RESULTS

Bacterial adherence to hydrocarbons—The basic assay for bacterial adherence t hydrocarbons is illustrated in Fig. 1.

The assay was performed with varying volumes of 3 test hydrocarbons, n-hexadecane, n-octane and p-xylene. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbons (e.g. *E. coli*), the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested (e.g., *A. calcoaceticus* RAG-1), a 'creamy' upper layer was obtained following mixing.

Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was found to be a function of volume of hydrocarbon added. The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5 percent isopropanol resulted in immediate droplet coalescence and the concomitant release of extracted cells into the aqueous phase, thereby demonstrating that the bacteria had partitioned to the hydrocarbon:water interface.

Tables 1 and 2 summarize a number of experiments in which cell surface hydrophobicity of a variety of bacteria was measured by adherence to hexadecane. The majority of laboratory strains examined exhibited low affinity towards the test hydrocarbon. Among the bacterial strains exhibiting high affinity for hexadecane were hydrocarbon-degrading *Acinetobacter calcoaceticus* strains RAG-1, HO1-N, B5W str 71 and CDC 7828, and bacteria which do not metabolize hydrocarbons: *Serratia marcescens, Staphylococcus aureus* and *Streptococcus pyogenes.*

Pseudomonas aeruginosa (which can utilize hexadecane as sole carbon and energy source) exhibited no significant affinity towards any of the test hydrocarbons when grown to late exponential phase in nutrient broth (Fig. 2). Since adherence in this organism might have required the presence of hydrocarbon during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane (Fig. 2). The affinity of alkane-grown *P. aeruginosa* towards hexadecane did increase somewhat (e.g., 32 percent of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane). In contrast, *A. calcoaceticus* RAG-1 exhibited much higher affinity when grown on hexadecane (93 percent of the cells could be removed by as little as 0.05 ml hexadecane).

Role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane

Acinetobacter calcoaceticus RAG-1 adheres avidly to hydrocarbons.[32,34] A spontaneous mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells which did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Fig. 3).

Mutant MR-481 resembled the wild type in the following characteristics: (a) colonial and cell morphology, (b) sensitivity to two bacteriophages, ap2 and ap3[26] which are specific for *A. calcoaceticus* RAG-1 strains, (c) production of extracellular emulsifying activity during growth on ethanol as sole carbon and energy source,[22] (d) doubling time on brain heart infusion broth (38 min at 30°C with aeration) and (e) agglutinability by antibodies raised against RAG-1 cells.[1]

TABLE 1—Adherence of Bacteria to Hexadecane

Strain	Source	Growth Medium[a]	Adherence to Hexadecane, pct[b]
Acinetobacter calcoaceticus RAG-1	This laboratory	BHI	97
Bacillus cereus	Local isolate	NB	1
Bacillus subtilis 168	E. Z. Ron	NB	0
Enterobacter aerogenes CDC 659/$_{66}$	E. Z. Ron	NB	9
Escherichia coli B	E. Z. Ron	NB	0
Escherichia coli K12	E. Z. Ron	NB	2
Escherichia coli J5 (rough)	E. Z. Ron	NB	11
Micrococcus lysodeikticus (ATCC 4698)	I. Friedberg	NB	0
Myxococcus xanthus	This laboratory	CT	35
Proteus mirabilis S1959	S. Rottem	NB	0
Pseudomonas aeruginosa PAS279	J. Shapiro	NB	0
Serratia marcescens	R. Zack	NB	96
Staphlococcus albus	R. Zack	NB	0
Staphlococcus aureus	R. Zack	NB	91
Streptococcus pyogenes M5	I. Ofek	BHI	84

[a] Cells were harvested at the end of the exponential phase, washed twice and suspended in PUM buffer to an initial absorbence at 400 nm of 1.1-1.6.

[b] Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

TABLE 2—Adherence of *Acinetobacter* Strains to Hexadecane

Strain Designation	Source	Growth on Hexadecane[a]	Adherence to Hexadecane, pct[b]
RAG-1 (ATCC 31012)	This laboratory	+	82
HO1-N	W. R. Finnerty	+	99
BD4	E. Juni	+	4
BD413	E. Juni	+	0
B5W str 71 (ATCC 15149)	K. Bryn	+	84
CDC 7827 (ATCC 17959)	K. Bryn	+	86
B5W (ATCC 17903)	K. Bryn	−	0
ATCC 17906	K. Bryn	−	52
ATCC 17977	K. Bryn	−	7
ATCC 17985	K. Bryn	−	41

[a] Growth was examined on PUM agar in the presence of hexadecane vapors.

[b] Adherence was measured on cells grown to stationary phase on BHI broth; the standard adherence assay was performed with 0.05 ml hexadecane.

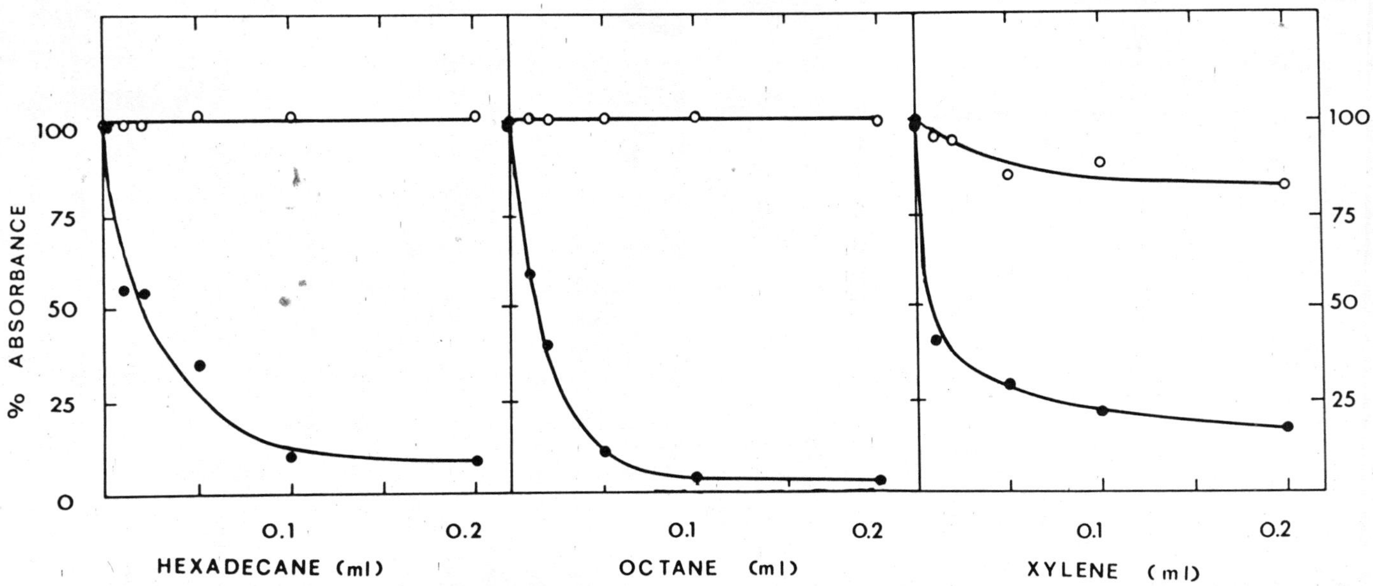

FIG. 1—Adherence of *Eschericia coli* B (○) and *Acinetobacter calcoaceticus* RAG-1 (•) to hydrocarbons. Cells were harvested [illegible] the end of the exponential growth phase, washed twice in PUM buffer and then resuspended to an initial A_{400} of 1.2-1.4. The adherence assay was performed as described in Materials and Methods. The ordinate is the percentage of initial absorbence of the cell suspension.

It should be emphasized that in all growth experiments inocula were prepared in the same manner as was described for measuring adherence in Fig. 3. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 4). Following a 2 hr lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew whereas the non-adherent mutant MR-481 failed to grow for at least 54 hr (Fig. 5). Growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells.

Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since attached bacteria were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Fig. 6).

Effect of emulsan on the growth of MR-481 on hexadecane

A. calcoaceticus RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan.[3,28,30,45] It was of interest to examine the effect of a highly purified preparation of emulsan (see Materials and Methods) on the growth of RAG-1 and the non-adherent mutant MR-481 on hexadecane (Fig. 7). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481.

However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both

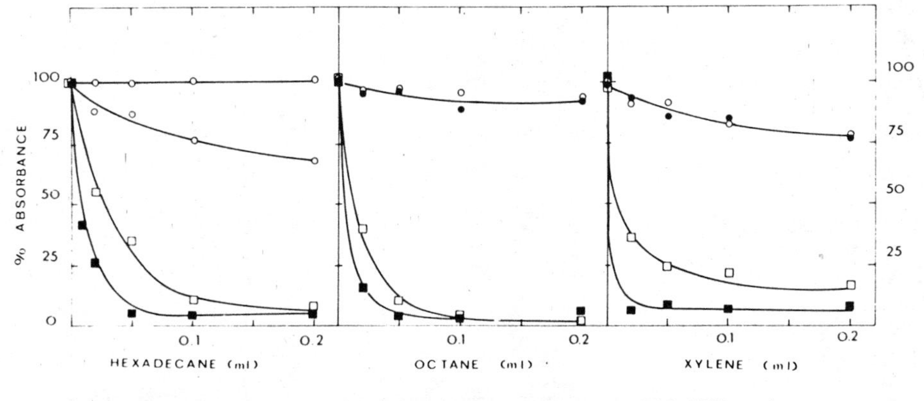

FIG. 2—Adherence of *Psuedomonas aeruginosa* PAS 279 and *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons. The experiment was performed as described in Fig. 1 with cells harvested at the end of the exponential growth phase: *P. aeruginosa*, NB medium (○), P-hexadecane medium (•); *A. calcoaceticus*, NB medium (□), hexadecane medium (■).

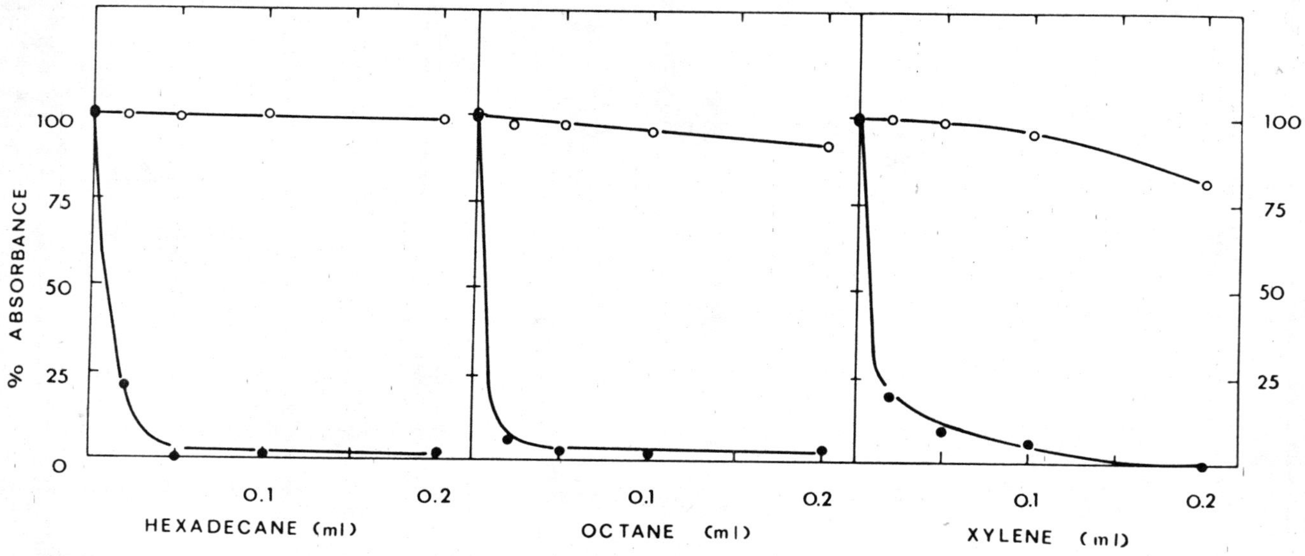

FIG. 3—Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth washed twice and resuspended in PUM buffer to an A_{400} = 1.45 to 1.50. To 1.2 ml of suspended RAG-1 (•) or MR-481 (○) cells were added to various volumes of test hydrocarbon and the mixtures vortexed for 120 sec. Following phase separation the turbidity of the lower aqueous phase was measured. The ordinate is the percent of initial absorbence of the cell suspension.

emulsan and hexadecane began after about 6 hr, whereas neither component alone supported growth for 54 hr. At the weight ratio of hexadecane: emulsan used, 1,925:1, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Fig. 8). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic or phenotypic

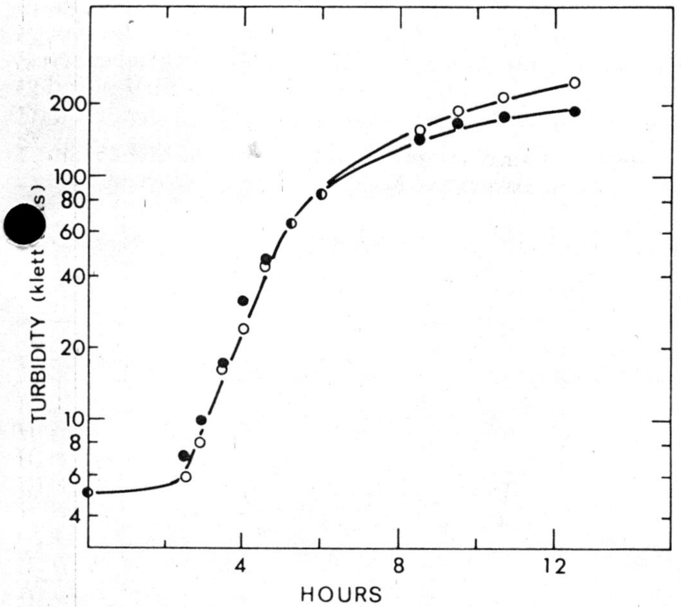

FIG. 4—Growth of RAG-1 and MR-481 in acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice and resuspended in PUM buffer supplemented with 0.2 percent sodium acetate. RAG-1 (•) and MR-481 (○) cells were inoculated into 4.2 ml acetate medium in Klett tubes to an initial turbidity of 5 Klett units.

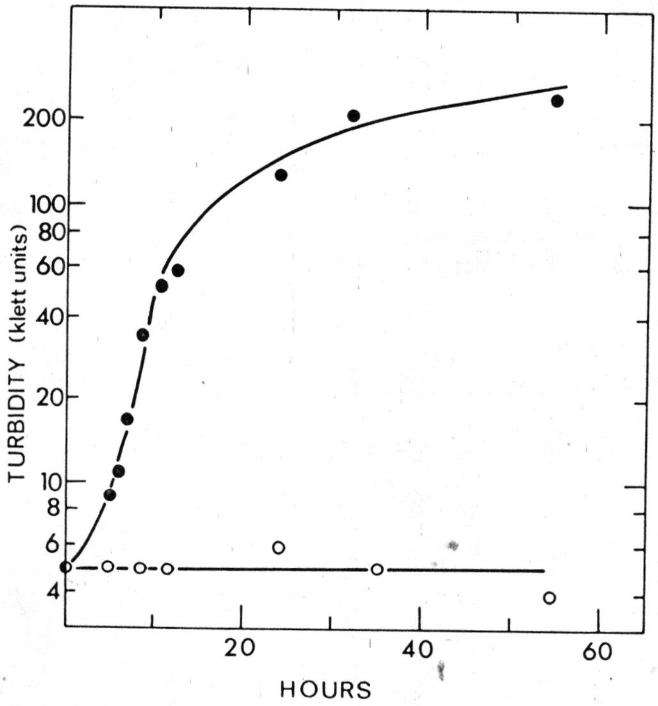

FIG. 5—Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml PUM buffer were inoculated with either RAG-1 (•) or MR-481 (○) to an initial turbidity of 5 Klett units. Tubes were overlayed with 0.5 ml hexadecane and incubated as described in Materials and Methods.

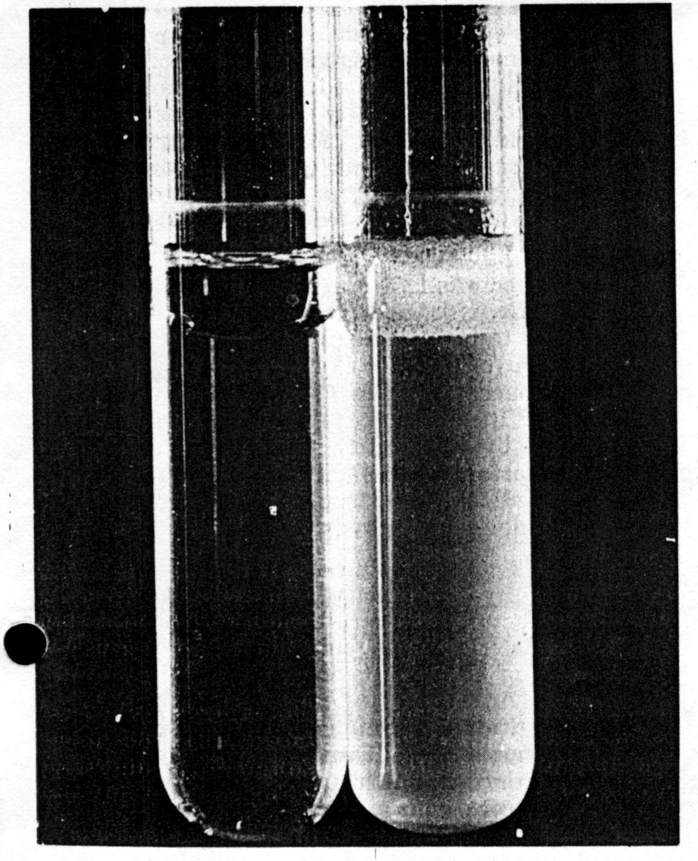

FIG. 6—Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. Tubes were photographed following 48 hr incubation under the conditions described in Fig. 3.

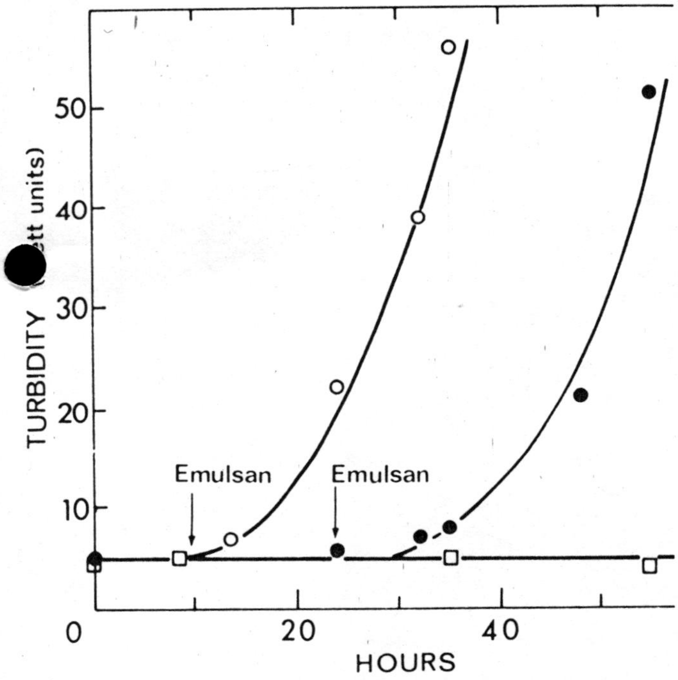

FIG. 8—Induction of growth of MR-481 on hexadecane by emulsan addition. Inocula preparation and incubation conditions were as described in Fig. 3. Emulsan was added to a final concentration of 50 μg/ml at 10 hr (○) or 24 hr (•). Tubes containing no emulsan served as controls (□).

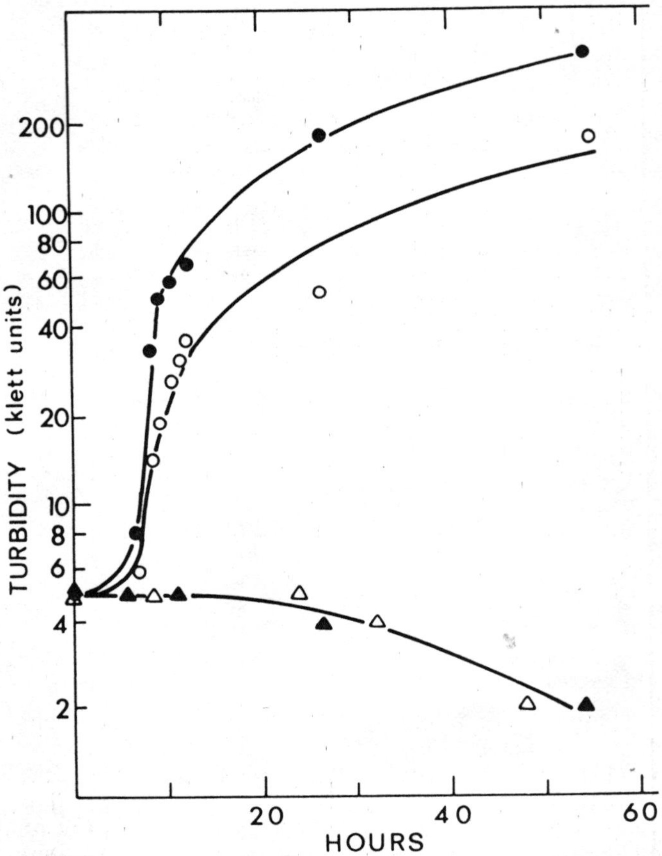

FIG. 7—Effect of emulsan on the growth of MR-481 on hexadecane. Inocula preparation and incubation conditions were as described in Fig. 3. RAG-1 (•) and MR-481 (○) were grown on hexadecane medium in the presence of 50 μg/ml emulsan. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg/ml emulsan, in the absence of hexadecane.

reversion of MR-481 from non-adherent to adherent cells. As shown in Table 3, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared to the initial inoculum grown in rich medium (Fig. 3).

RAG-1 cells which were harvested and washed following growth on hexadecane both in the presence and absence of emulsan, exhibited high affinity towards hexadecane (Table 3). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

DISCUSSION

Mudd and Mudd[21] reported over 50 years ago that certain microorganisms tend to concentrate at oil:water interfaces. Although this observation has been frequently corroborated during studies on the growth of microorganisms on liquid hydrocarbons,[13,15,19,20,22,23] there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Certain investigators have argued that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets.[5,43,44] *A. calcoaceticus* RAG-1 is an interesting microorganism in

TABLE 3—Adherence of RAG-1 and MR-481 Following Growth on Hexadecane

Strain	Turbidity (A_{400})[a]		Percent Adherence
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR-481[c]	1.43	1.36	4.9

[a] Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested following 54 hr growth on hexadecane medium, washed twice and resuspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested following 54 hr growth on hexadecane medium in the presence of 50 μg/ml emulsan, washed twice and resuspended in PUM buffer.

this regard because it both adheres avidly to hydrocarbons[32,33,34] and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized.[3,28,30,45]

Isolation of a mutant strain of *A. calcoaceticus* RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The non-adherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were excluded by the following data: (1) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (2) MR-481 was able to grow on hexadecane medium following addition of an emulsifier; and (3) MR-481 cells were able to grow on solid medium supplemented with hexadecane vapors as sole carbon and energy source. We conclude from these data that adherence is a prerequisite for growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce a potent extracellular emulsifying agent which enables growth of non-adhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon.[16,29,35] At the low inoculum employed (5×10^7 cells/ml) the concentration of emulsan which the cells produce is too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of non-adherent bacteria on hydrocarbons. The poor adherence of *P. aeruginosa* PAS 279 to hydrocarbon (Fig. 2) suggests that its growth, similar to that of MR-481, depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments, conditions of low cell density and limited emulsification prevail; thus, the growth of poorly-adhering *pseudomonas* strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation[5,14,43,44] probably does not reflect growth in the open environment, where emulsifier and dispersed hydrocarbon would tend to diffuse away from the cell.

The adherence to hydrocarbon of bacteria which do not metabolize hydrocarbons (Tables 1 and 2) can be explained in terms of general cell surface hydrophobicity.[21,32] *S. pyogenes,* for example, has been reported to adhere to buccal epithelial cells through hydrophobic interactions.[2] Its adherence to hydrocarbons may thus be mediated by such interactions. Similarly, hydrophobic cell surface components which enable *Serratia* to partition at the air:water interface[4] may also promote their adherence to hydrocarbons as observed in this study.

Bacterial hydrophobicity has been cited as an important factor in several biological phenomena, including bacterial pathogenicity,[42] and in adherence and growth of bacteria on various hydrophobic surfaces.[2,19,24,35] Adherence to hydrocarbons provides a simple quantitative technique for measuring cell surface hydrophobicity and can be used for the selection and isolation of non-adhering mutants. Adherence of RAG-1 to hydrocarbons may reflect a general phenomenon in microbiology: A biological advantage is conferred on microbial cells which can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface which provides nutrition and enables growth.

REFERENCES

1. Bayer, E. A., E. Rosenberg and D. Gutnick. 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. J. Gen Microbiol. *127:* 295-300.

2. Beachey, E. H., W. A. Simpson and I. Ofek. 1980. Interaction of surface polymers of *Streptococcus pyogenes* with animal cells, p. 389-405. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.). Microbial Adhesion to Surfaces. Society of Chemical Industry, London.

3. Belsky, I., D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: Determination of emulsifier-bound fatty acids. FEBS Lett. *101:* 175-178.

4. Blanchard, D. C. and L. D. Syzdek. 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. *23:* 389-400.

5. Chakravarty, M., H. D. Singh and J. N. Baruah. 1975. A kinetic model for microbial growth on liquid hydrocarbons. Biotech. Bioeng. *17:* 399-412.

6. Daniels, S. L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces, pp. 7-58. *In* G. Bitton and K. C. Marshall (Eds.). Adsorption of Microorganisms to Surfaces. John Wiley & Sons, New York.

7. Dexter, S. C., J. D. Sullivan, Jr., J. Williams, III, and S. W. Watson. 1975. Influence of substrate wettabil-

ity on the attachment of marine bacteria to various surfaces. Appl. Microbiol. *30:* 298-308.

8. Dworkin, M. 1973. Cell-to-cell interactions in the myxobacteria. Symp. Soc. Gen. Microbiol. *23:* 125-142.

9. Fletcher, M. and G. I. Loeb. 1979. The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. *37:* 67-72.

10. Foster, J. W. 1962. Bacterial oxidation of hydrocarbons, pp. 241-261. *In:* Oxygenases. O. Hayaishi (Ed.), Academic Press, New York.

11. Gersen, D. F. and D. Sheer. 1980. Cell surface energy, contact angles and phase partition. III Adhesion of bacterial cells to hydrophobic surfaces. Biochem. Biophys. Acta *602:* 506-510.

12. Gibson, D. T. 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. *I:* 199-223.

13. Gutnick, D. L. and E. Rosenberg. 1977. Oil tankers and pollution: A microbiological approach. Ann. Rev. Microbiol. *31:* 379-396.

14. Hisatsuka, K., T. Nakahara, Y. Minoda and K. Yamada. 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agr. Biol. Chem. *39:* 999-1005.

15. Kirschner Zilber, I., E. Rosenberg and D. Gutnick. 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on n-tetracosane. Appl. Environ. Microbiol. *40:* 1086-1093.

16. Lankford, C. E., J. R. Walker, J. B. Reeves, N. H. Nabbut, B. R. Byers and R. J. Jones. 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s). ("Schizokinen"). J. Bacteriol. *91:* 1070-1079.

17. Latham, M. J. 1980. Adhesion of rumen bacteria to plant cell walls, p. 339-350. *In:* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (Eds.), Microbial Adhesion to Surfaces. Society of Chemical Industry.

18. Lippincott, J. A. and B. Lippincott. 1980. Microbial adherence in plants, p. 375-398. *In:* E. H. Beachey, (Ed.), Bacterial Adherence. Chapman and Hall, New York.

19. Marshall, K. C. 1976. Interfaces in Microbial Ecology. Harvard University Press. Cambridge, Mass.

20. McLee, A. G. and S. L. Davies. 1972. Linear growth of a *Torulopsis* sp. on n-alkanes. Can. J. Microbiol. *18:* 315-319.

21. Mudd, S. and E. B. H. Mudd. 1924. The penetration of bacteria through capilary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. *40:* 633-645.

22. Nakahara, T., L. E. Erickson and J. R. Gutierrez. 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotech Bioeng. *19:* 9-25.

23. Neufeld, R. J., J. E. Zajic and D. F. Gerson. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. Appl. Environ. Microbiol. *39:* 511-517.

24. Ofek, I. and E. H. Beachey. 1980. General concepts and principles of bacterial adherence in animals and man. p. 1-31. *In:* E. H. Beachey (Ed.). Bacterial Adherence, Chapman and Hall, London and New York.

25. Perers, L., L. Andaker, L. Edebo, O. Stendahl and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta. Path. Microbiol. Scand. Sect. B *85:* 308-316.

26. Pines, O. and D. L. Gutnick. 1981. Relationship between phage resistance—emulsan production and interaction of phages with the cell surface of *Acinetobacter calcoaceticus* RAG-1. Arch. Microbiol. *130:* 129-133.

27. Ratledge, C. 1978. Degradation of aliphatic hydrocarbons, p. 1-46. *In:* R. J. Watkinson (Ed.). Developments in Biodegradation of Hydrocarbons—I. Applied Science Publishers, London.

28. Reisfeld, A., E. Rosenberg and D. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Environ. Microbiol. *24:* 363-368.

29. Rosenberg, E., K. H. Keller and M. Dworkin. 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. J. Bacteriol. *129:* 770-777.

30. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D. L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. *37:* 402-408.

31. Rosenberg, M. 1981. Bacterial adherence to polystryene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. *42:* 375-377.

32. Rosenberg, M., D. Gutnick and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. *9:* 29-33.

33. Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg and I. Ofek. 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. *33:* 29-33.

34. Rosenberg, M., E. Rosenberg and D. Gutnick. 1980. Bacterial adherence to hydrocarbons, p. 541-542. *In:* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.). Microbial Adhesion to Surfaces. Society of Chemical Industry, London.

35. Rosenberg, M. and E. Rosenberg. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. *148:* 51-57.

36. Shapiro, L. 1976. Differentiation in the caulobacter cell cycle. Ann. Rev. Microbiol. *30:* 377-407.

37. Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén and T. Wadström. 1978. Differences in hydrophobic surface characteristics of procine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. *22:* 462-472.

38. Stanier, R. Y. 1942. The cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. *6:* 143-196.

39. Starr, M. P. and J. C. C. Huang. 1972. Physiology of the *Bdellovibrios. In:* Advances of Microbiological Physiology. *8:* 21-261. A. H. Rose and D. W. Tempest (Eds.). Academic Press, London.

40. Sundarrj, C. P. and J. V. Bhat. 1972. Breakdown of chitin by *Cytophaga johnsoni.* Arch. fur Mikrobiologie *85:* 159-167.

41. Varon, M. and M. Shilo. 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *B. Bacteriovorus.* J. Bacteriol. *95:* 744-753.

42. van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. *32:* 19-39.

43. Velankar, S. K., S. M. Barnett, C. W. Houston and A. R. Thompson. 1975. Microbial growth on hydrocarbons—some experimental results. Biotech. Bioeng. *17:* 241-251.

44. Wang, D. I. C. and A. Ochoa. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentation and relationships to specific growth rates. Biotech. Bioeng. *14:* 345-360.

45. Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. *37:* 414-420.

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MICROBIAL ADHESION TO SURFACES

Editors:

R. C. W. BERKELEY
Department of Bacteriology, University of Bristol
J. M. LYNCH,
Agricultural Research Council
J. MELLING
Centre for Applied Microbiology and Research, Porton Down
P. R. RUTTER
Physical Sciences Branch, B.P. Research Centre
and
B. VINCENT
Department of Physical Chemistry, University of Bristol

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the radioactivity of the resin and the eluate. 316 isolates from the four stations, about half the number of isolates deriving from the surface microlayers, the other half from the corresponding subsurface waters were assessed for their hydrophobicity and 25 of them were characterised as to relative net surface charge.

The results revealed a positive correlation between the degree of accumulation of bacteria at the surface and their hydrophobicity. At three stations a higher portion was found of hydrophobic bacteria inhabiting the surface compared to the subsurface water.

The fourth station, where no bacterial enrichment was found at the surface no difference in the degree of hydrophobicity between bacteria collected from the surface and from the subsurface was detected. Surface bacteria with a low degree of hydrophobicity adhered to the surface as a result of physico-chemical factors other than hydrophobicity, for example, charge-charge interactions.

None of the bacteria possessed a high negative net surface charge together with a high degree of hydrophobicity. All anion exchange resin ratios, whether high or low, corresponded [illegible] low cation resin exchange ratios indicating that the bacteria had a net surface charge ranging from around zero to strongly negative.

28.15 BACTERIAL ADHERENCE TO HYDROCARBONS

M. Rosenberg, E. Rosenberg and **D. Gutnick**, Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

The hydrophobic nature of the external surface of the bacterial cell has been implicated in a variety of biological phenomena involving bacterial adherence. One interesting example is the growth of micro-organisms on solid and liquid paraffins by direct contact with the bulk hydrocarbon phase. In this report, a simple, rapid assay for studying the adherence of bacteria to liquid hydrocarbon was developed in order to answer the following questions: (1) Is the ability to adhere to hydrocarbons restricted to hydrocarbon-degrading bacteria? (2) Are there significant differences among hydrocarbon-degrading bacteria with respect to their affinity towards hydrocarbon? (3) Do hydrocarbon-degraders adhere specifically to those hydrocarbons which they are able to metabolise? (4) Can changes in physiological conditions affect bacterial adherence to hydrocarbons?

The assay was performed as follows: various volumes of test hydrocarbon (n-hexadecane, [illegible]ctane or p-xylene) were added to turbid aqueous bacterial suspensions and the mixtures were uniformly agitated for two minutes. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbon, the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested, a 'creamy' upper layer was obtained following mixing. Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was found to be a function of volume of hydrocarbon added. In some cases, addition of 0.2 ml test hydrocarbon to 1.2 ml bacterial suspension resulted in the removal of over 99% of the cells from the aqueous phase, as evidenced both by loss in turbidity and decrease in total cell number.

The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5% isopropanol resulted in immediate droplet coalescence and the concommitant release of extracted cells into the aqueous phase.

Adherence of Bacteria which Do Not Metabolise Hydrocarbons

In most experiments described here, bacteria were grown aerobically in nutrient broth and harvested in mid to late exponential phase. The majority of strains tested under these growth conditions exhibited no significant affinity towards the test hydrocarbons. Non-adherent strains included the following Gram-positive and Gram-negative bacteria: *Bacillus subtilis, Micrococcus lysodeikticus, Staphylococcus albus, Escherichia coli B* and *K-12, Enterobacter aerogenes,* and *Serratia marcescens.*

In contrast, *Staphylococcus aureus* and stationary phase *Serratia marescens* cells exhibited high affinity for the test hydrocarbons. Recently it was also shown that *Streptococcus pyogenes* strain *M-5* grown in brain heart infusion broth also exhibits high affinity towards hydrocarbons (A. Perry and M. Rosenberg, unpublished results). These data show that adherence to hydrocarbons is not an exclusive property of hydrocarbon-degrading micro-organisms.

Adherence of Hydrocarbon-Degrading Bacterium to Non-Metabolisable Hydrocarbons

Acinetobacter calcoaceticus RAG-1, a hydrocarbon-degrading micro-organism which has been studied in this laboratory for several years, adhered strongly to all three test hydrocarbons, despite its inability to metabolise octane and xylene. *RAG-1* was also observed to adhere to a variety of non-wettable solid surfaces, such as polystyrene Petri dishes and siliconised glass. These data indicate that the adherence of *RAG-1* to hydrocarbon is not specific for metabolisable substrates, and that a general hydrophobic interaction, rather than a specific recognition mechanism may be involved in the adherence of *RAG-1* to hydrocarbon.

Non-adherence of Hydrocarbon-degrading Bacterium

Pseudomonas aeruginosa (which can utilise hexadecane as sole carbon and energy source) exhibited no significant affinity towards any of the test hydrocarbons when grown to late exponential phase in nutrient broth. Since adherence in this organism might have required the presence of hydrocarbon during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane.

The affinity of alkane-grown *P. aeruginosa* did increase somewhat (that is 32% of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane). In contrast, *RAG-1* exhibited much higher affinity when grown under the same conditions (93% of the cells coul be removed by as little as 0.05 ml hexadecane). The large differences in relative affinities of *P. aeruginosa* and *RAG-1* to hydrocarbons, both after growth in nutrient broth and on haxadecane, suggest that even hydrocarbon-degrading bacteria may vary greatly in their ability to adhere to the bulk hydrocarbon plate.

Effect of Growth Conditions on Adherence

RAG-1 cells showed no affinity towards hexadecane when harvested from nutrient broth during early exponential phase, whereas late exponential and stationary phase cells adhered strongly to each of the test hydrocarbons. A similar but less-pronounced increase in affinity with culture age was observed when *RAG-1* was grown in defined medium with ethanol as sole carbon and energy source. These results suggest that culture age may have a greater effect on the development of cell surface hydrophobicity than growth medium.

The mechanisms responsible for the cell surface changes in *RAG-1* as well as the specific cell surface components which participate in the hydrophobic interactions described here are currently under investigation.

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Adherence of *Acinetobacter calcoaceticus* RAG-1 to Human Epithelial Cells and to Hexadecane

MEL ROSENBERG,[1]* ALEX PERRY,[2] EDWARD A. BAYER,[1] DAVID L. GUTNICK,[1] EUGENE ROSENBERG,[1] AND ITZHAK OFEK[2]

Department of Microbiology, George S. Wise Faculty of Life Sciences,[1] and Department of Human Microbiology, Sackler School of Medicine,[2] Tel Aviv University, Ramat Aviv, Israel

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The ability of *Acinetobacter calcoaceticus* RAG-1 to adhere to human epithelial cells was investigated and compared with its ability to adhere to a test hydrocarbon (hexadecane). RAG-1, a microorganism originally isolated for growth on hydrocarbon, adhered to epithelial cells when grown under conditions which promote its adherence to hexadecane; similarly, RAG-1 cells adhered poorly to epithelial cells when grown under conditions which cause the cells to possess low affinity towards hexadecane. A mutant derived from RAG-1, MR-481, deficient in its ability to adhere to hydrocarbon, was similarly unable to adhere to epithelial cells. RAG-1 adherence to epithelial cells was not blocked by a number of sugars tested. *Streptococcus pyogenes*, whose adherence to epithelial cells has been previously attributed to hydrophobic interactions, was also able to adhere to hexadecane. Results suggest that hydrophobic interactions mediate adherence of the strains studied to both epithelial cells and hydrocarbon.

There is increased interest in the investigation of adherence of various microorganisms in their natural environment and the mechanisms involved (2). In such studies. bacteria are routinely assayed under laboratory conditions for adherence to those surfaces which they normally colonize in nature. Bacteria, however, may also adhere to surfaces which they do not normally encounter. Marine bacteria, for example, can adhere to and colonize plastic surfaces (4, 6, 7). Similarly, a recently developed assay revealed that adherence to hydrocarbon droplets is not restricted to hydrocarbon-degrading microorganisms (15). Preliminary observations indicated that *Streptococcus pyogenes* M-5, a strict colonizer of epithelial cells, also adhered to hydrocarbon. It was therefore of interest to see whether a hydrocarbon-degrading *Acinetobacter* strain (14, 16), whose adherence to hydrocarbon has been studied previously (15), would adhere to epithelial cells that are normally colonized by pathogenic bacteria but rarely, if ever, inhabited by *Acinetobacter* species.

MATERIALS AND METHODS

Bacterial growth conditions. *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) was grown in either nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl or brain heart infusion broth (Difco); cells were grown with shaking at 30°C and harvested as indicated. *S. pyogenes* M-5 (H type 5) was grown at 37°C in brain heart infusion broth under static conditions and harvested at 48 h.

Epithelial cells. Epithelial cells were obtained as described previously (12) by scraping the buccal mucosa of several healthy donors with wooden sticks. The cells were washed twice and suspended in phosphate-buffered saline to a concentration of 10^5 cells per ml.

Adherence to hexadecane. A rapid and simple assay for bacterial adherence to hexadecane has recently been reported (15) and is described here in brief. Harvested bacteria were washed twice and resuspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, and distilled water to 1,000 ml), pH 7.1. To 1.2 ml of a turbid bacterial suspension (absorbance at 400 nm, 1.4 to 1.6) in acid-washed test tubes, 0.2 ml of hexadecane was added. After 10 min of preincubation at 30°C, the two phases were mixed under controlled conditions for 120 s. After the phases were allowed to separate, the aqueous phase was carefully removed, and its light absorbance was measured by using a Gilford model 240 spectrophotometer. The fraction of adherent cells was taken as the percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared with that of the original suspension.

Bacterial adherence to epithelial cells. An epithelial cell suspension (0.5 ml) was mixed with an equal volume of bacterial suspension (2×10^{10} cells per ml), conditions which enable optimal adherence. The mixtures were rotated end over end for 30 min at

37°C (12). The epithelial cells were separated from the nonadherent bacteria by filtration through polycarbonate filters (12-μm pore size) (Nuclepore Corp., Pleasanton, Calif.), washed extensively with phosphate-buffered saline and smeared onto glass microscope slides. The preparations were stained following drying with Hemacolor rapid stain (Merck & Co., Inc., Rahway, N.J.) and examined with a bright-field microscope. Adherence was recorded as the percentage of the epithelial cells bearing 100 or more attached bacteria. Clumping of bacteria was not observed in either adherence assay.

Addition of sugars (of the highest purity obtainable) to the adherence assay was performed as described previously (12).

Isolation of a nonadhering mutant of *A. calcoaceticus* RAG-1. Strain MR-481, a mutant deficient in its ability to adhere to hydrocarbon. was isolated by repeated transfers of RAG-1 cells which remained in the aqueous phase after mixing with *n*-octane. This mutant resembled the wild type in phage sensitivity, agglutination by specific antibodies, and production of emulsifying activity during growth on ethanol as sole carbon source (16).

RESULTS

S. pyogenes M-5 is a pathogen with high affinity for epithelial cells (1, 11). In routine tests, over 30% of the epithelial cells scored were covered by 100 or more bacteria. Strain M-5 was found to adhere to three test hydrocarbons examined (hexadecane, octane, and *p*-xylene). Adherence of *S. pyogenes* M-5 to hexadecane is shown in Fig. 1.

A. calcoaceticus RAG-1, a hydrocarbon-degrading bacterium, has previously been found to adhere avidly to hydrocarbons (15) and to other hydrophobic surfaces, such as polystyrene (M. Rosenberg, unpublished data). The adherence of RAG-1 to hexadecane increased greatly with culture age (Fig. 2). Early-log-phase cells grown on nutrient broth exhibited very low affinity towards hexadecane, whereas late-log-phase cells adhered to the hydrocarbon. It was of interest to see whether a similar dependence on culture age could be observed in RAG-1 adherence to epithelial cells. Adherences of early- and late-log-phase cells of RAG-1 to buccal epithelial cells and hexadecane were compared (Fig. 3). RAG-1 cells harvested during late exponential phase adhered with high affinity to both epithelial cells and hexadecane droplets, whereas early-exponential-phase cells exhibited low affinity towards both surfaces, even at the high ratio of bacterial to epithelial cells employed.

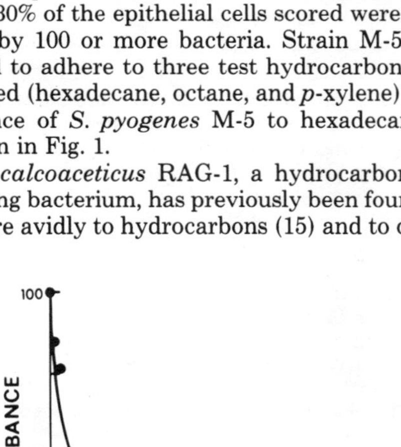

FIG. 1. *Adherence of S. pyogenes M-5 to hexadecane. Cells were grown on brain heart infusion broth to stationary phase (48 h), washed twice, and suspended in PUM buffer. Adherence to hexadecane was carried out as described in the text. Results are expressed as the percentage of the initial absorbance at 400 nm of the aqueous suspension (after mixing) as a function of hydrocarbon volume added.*

Attempts were made to inhibit RAG-1 adherence to epithelial cells by addition of monosaccharides (12). None of the sugars tested (D-mannose, D-glucose, methyl-β-D-glucoside, D-glucosamine, *N*-acetyl-D-glucosamine, D-galactose, methyl-α-D-galactoside, maltose, and L-arabinose) blocked RAG-1 adherence to epithelial cells at concentrations as high as 25 mg/ml, suggesting that RAG-1 adherence to epithelial

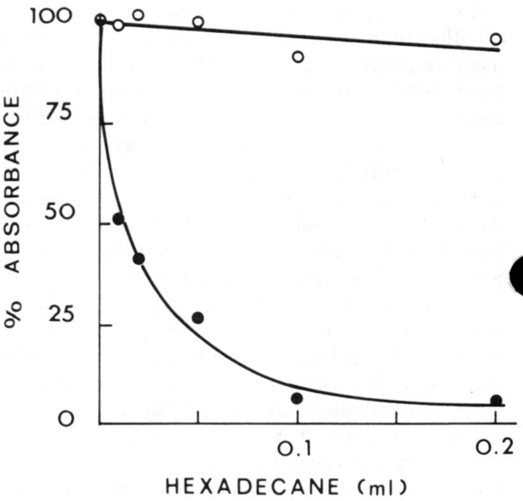

FIG. 2. *Adherence of early- (○) and late- (●) log-phase A. calcoaceticus RAG-1 cells to hexadecane. RAG-1 cells were grown in nutrient broth containing 0.5% NaCl and harvested from the same culture at 15 Klett units (early logarithmic phase) and at 65 Klett units (late logarithmic phase) as measured in a Klett-Summerson colorimeter fitted with a green filter. Cells were washed and assayed for adherence to hexadecane as described in the text. Results are expressed as the percentage of the initial absorbance at 400 nm of the aqueous suspension (after mixing) as a function of hydrocarbon volume added.*

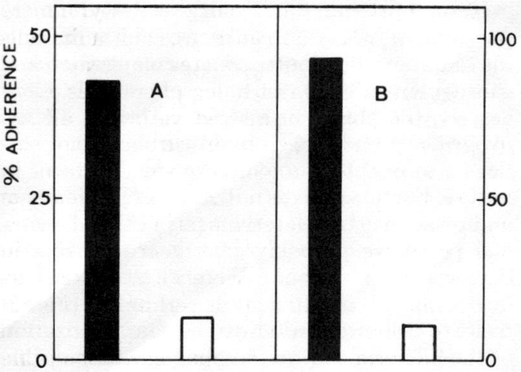

FIG. 3. *Adherence of A. calcoaceticus RAG-1 to hexadecane and epithelial cells. RAG-1 cells, grown in nutrient broth containing 0.5% NaCl, were harvested from the same culture at 20 Klett units (early logarithmic phase; open bars) and 80 Klett units (late logarithmic phase; black bars). Cells were washed and assayed for adherence to both hexadecane and epithelial cells as described in the text. Adherence to epithelial cells is shown as the percentage of epithelial cells with 100 or more attached bacteria (A). Adherence to hexadecane is shown as the percentage of bacteria removed from the aqueous phase (after mixing) in the presence of 0.2 ml of hexadecane (B).*

cells is not mediated by recognition of monosaccharide residues.

Isolation of a mutant of RAG-1 deficient in its ability to adhere to hydrocarbon enabled further studies to be carried out on the above correlation between RAG-1 adherence to epithelial cells and to hexadecane. This mutant, referred to as MR-481, adhered neither to epithelial cells nor to hexadecane, even when grown under conditions which enhanced adherence of wild-type RAG-1 cells to both surfaces. In a representative experiment, RAG-1 and mutant MR-481 cells were grown to stationary phase in brain heart infusion broth, harvested, washed in buffer, and tested for adherence (Fig. 4). Although 100% of the wild-type cells adhered to hexadecane under these conditions, only 2.6% of the mutant cells adhered to the test hydrocarbon. Similarly, RAG-1 cells adhered with an extremely high affinity to epithelial cells (66% of the epithelial cells scored were covered by 100 or more bacteria), whereas only 3% of the epithelial cells incubated with MR-481 cells were covered by 100 or more bacteria.

DISCUSSION

In a previous report (15), we described a simple, quantitative method for measuring bacterial adherence to liquid hydrocarbons. Although most strains examined showed little or no affinity for the test hydrocarbons (hexadecane, octane, and xylene), others were highly adherent and were thus increasingly removed from the lower aqueous phase upon rapid mixing with increasing volumes of hydrocarbon. Adherent cells were observed microscopically on the surface of the hydrocarbon droplets. Addition of surfactants, such as isopropanol, resulted in the release of adherent cells back into the aqueous phase and recovery of most of the initial turbidity, demonstrating that lysis did not occur in the assay procedure. The adherence to hydrocarbon found for various strains (*Serratia marcescens*, *Staphylococcus aureus*, and *Escherichia coli* J-5) correlated with previous reports of hydrophobic interactions involving these and similar bacterial strains (5, 9, 10) and suggested that adherence of bacteria to hydrocarbon provides a simple method for measuring cell surface hydrophobicity.

The present study relates to the ability of bacteria to adhere to surfaces which they do not colonize under natural conditions. Certain pathogenic microorganisms, such as streptococci and staphylococci (15), can adhere to hydrocarbons, although they almost exclusively colonize epithelial cells. Conversely, an oil-degrading *Acinetobacter* strain, which was originally isolated for ability to degrade petroleum (14), was shown in the present report to adhere avidly to epithelial cells. *Acinetobacter* species, although sometimes isolated in clinical studies, are probably not pathogenic, and rarely, if ever, colonize buccal epithelial cells (8).

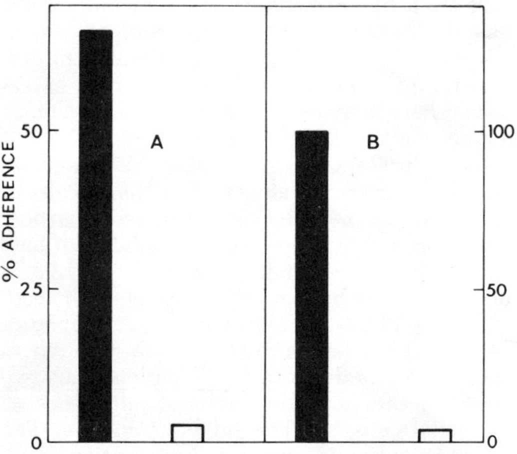

FIG. 4. *Adherence of A. calcoaceticus RAG-1 and mutant MR-481 to hexadecane and epithelial cells. Cells of RAG-1 and mutant MR-481 were inoculated 1:1,000 and harvested at stationary phase after 18 h. Determination of adherence of RAG-1 (black bars) and MR-481 (open bars) to epithelial cells (A) and hexadecane (B) was as described in the legend to Fig. 3.*

Why then, should a given species of bacteria develop characteristics which enable it to adhere to surfaces with which it rarely comes into contact? The simplest explanation is that the target surfaces studied here share common characteristics, thus enabling adherence of bacteria by a common mechanism. Results presented here show that a correlation exists between *A. calcoaceticus* RAG-1 adherence to epithelial cells and adherence to hexadecane. This correlation was strengthened by the inability of a mutant deficient in adherence to hexadecane to bind to epithelial cells.

If a common mechanism is indeed involved, then hydrophobic interactions between the bacteria and the surfaces studied here may play an important role. Hydrophobic interactions contribute to the adherence of pathogenic bacteria to various host cells (9, 13, 17) and the adherence of bacteria to hydrophobic plastic surfaces (4, 6, 7). Bacterial hydrophobicity has also been shown to be an important factor in phagocytic attachment (19). In a previous report (15), it was suggested that adherence of RAG-1 to hydrocarbon is mediated by a general hydrophobic interaction, rather than specific recognition of the hydrocarbon substrate; RAG-1 adherence to epithelial cells may involve similar interactions between hydrophobic sites on the bacterial and epithelial cell surfaces.

A similar case may be made for streptococcal adherence. The lipid moiety of lipoteichoic acid on the streptococcal surface was found to mediate adherence of these organisms to epithelial cells by a hydrophobic interaction mechanism (3, 11). Tylewska et al. reached similar conclusions, using phenyl-Sepharose columns (18). Adherence of streptococci to hexadecane, as reported here, may involve these same covalently bound lipids.

In the present report, we have shown correlations between the ability of bacterial cells to adhere to epithelial cells and to hydrocarbon. Similar correlations between the ability of bacteria to bind to animal cells and surface hydrophobicity have been reported, using other techniques (9, 13, 17–19). Nevertheless, further studies with other bacterial strains are required to determine whether the participation of hydrophobic interactions in bacterial adherence to epithelial cells is a general phenomenon. The use of bacterial adherence to hydrocarbon as an indication of bacterial hydrophobicity has certain advantages over other methods: (i) the surface to which the bacteria adhere (i.e., liquid hydrocarbons) is well defined, (ii) intricate equipment is not required, and (iii) quantitative results are rapidly and easily obtained.

Results presented here suggest that the mere potential of bacteria to adhere to epithelial cells may not be sufficient to initiate colonization and subsequent infection of mucosal surfaces. Conversely, the ability of a strict pathogen (i.e., *S. pyogenes*) to adhere to hydrocarbon is not sufficient to enable it to colonize such surfaces in nature. Factors such as nutritional requirements and resistance to deleterious physical and chemical pressures probably play a crucial role in bacterial colonization. Adherence to a given surface, although insufficient, is certainly a prerequisite to colonization. Thus, the characterization of the bacterial surface components responsible for bacterial adherence to hydrocarbon may help clarify some of the mechanisms through which microorganisms recognize and are recognized by the surfaces with which they interact.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Alkan, M., I. Ofek, and E. H. Beachey.** 1977. Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. Infect. Immun. **18:**555–557.
2. **Beachey, E. H.** 1980. Preface, p. 11–12. *In* E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall, London.
3. **Beachey, E. H., and I. Ofek.** 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. J. Exp. Med. **143:**759–771.
4. **Dexter, S. C., J. D. Sullivan, Jr., J. Williams III, and S. W. Watson.** 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl. Microbiol. **30:**298–308.
5. **Dyar, M. T.** 1948. Electrokinetical studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material, and some other surface properties. J. Bacteriol. **56:**821–834.
6. **Fletcher, M., and G. I. Loeb.** 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. **37:**67–72.
7. **Gerson, D. F., and D. Scheer.** 1980. Cell surface energy, contact angles and phase partition. III. Adhesion of bacterial cells to hydrophobic surfaces. Biochim. Biophys. Acta. **602:**506–510.
8. **Henrikson, S. D.** 1973. *Moraxella, Acinetobacter*, and the *Mimeae*. Bacteriol. Rev. **37:**522–561.
9. **Kihlstrom, E., and L. Edebo.** 1976. Association of viable and inactivated *Salmonella typhimurium* 395 MS and MR10 with HeLa cells. Infect. Immun. **14:**851–857.
10. **Kjelleberg, S., C. Lagercrantz, and T. Larsson.** 1980. Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. FEMS Microbiol. Lett. **7:**41–44.
11. **Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell.** 1975. Cell membrane binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. **141:**990–1003.

12. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature (London) **265:**623–625.
13. **Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tagesson.** 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Path. Microbiol. Scand. Sect. B **85:**308–316.
14. **Reisfeld, A., E. Rosenberg, and D. Gutnick.** 1972. Microbial degradation of crude oils: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. **24:**363–368.
15. **Rosenberg, M., D. L. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbon: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:**29–33.
16. **Rosenberg, E., A. Zuckerberg, C. Rubinowitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37:**402–408.
17. **Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22:**462–472.
18. **Tylewska, S. K., S. Hjertén, and T. Wadström.** 1979. Contribution of M protein to the hydrophobic surface properties of *Streptococcus pyogenes*. FEMS Microbiol. Lett. **6:**249–253.
19. **Van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32:**19–39.

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Role of Adherence in Growth of *Acinetobacter calcoaceticus* RAG-1 on Hexadecane

MEL ROSENBERG AND EUGENE ROSENBERG*

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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The high affinity of *Acinetobacter calcoaceticus* RAG-1 for liquid hydrocarbons permitted the isolation of a spontaneous nonadherent mutant, MR-481. Strain MR-481 exhibited no significant affinity for three test hydrocarbons, yet resembled the wild type in many properties, including production of the extracellular emulsifying agent emulsan. To study the role of adherence in growth on hydrocarbons, RAG-1 and MR-481 were compared for growth on hexadecane under conditions of limited agitation and at low initial cell densities. Adherent RAG-1 cells were able to grow rapidly under these conditions, whereas nonadherent MR-481 cells failed to grow for at least 54 h. However, the addition of emulsan either initially or at various times after inoculation enabled the nonadherent MR-481 cells to grow on hexadecane. Growth was not the result of reversion of MR-481 from nonadherent to adherent cells. The data demonstrate that adherence is a crucial factor in the growth of *A. calcoaceticus* RAG-1 on hexadecane in the absence of extracellular emulsification of the substrate.

The importance of cell adherence in the growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces (28). The initial step in the life cycle of *Bdellovibrio* cells is attachment to gram-negative bacteria (30, 33). Specific cell-to-cell interactions pervade the entire life cycle of myxobacteria (4, 29). Species-specific adhesion and tissue-specific adhesion are prerequisites for the successful colonization by microorganisms of animals (18) and plants (12). Microbial adhesion to surfaces has been the subject of several recent reviews (3, 11-13, 18).

In cases in which the carbon or energy source is a water-insoluble material such as cellulose or chitin, cell adhesion can facilitate growth (11, 29, 31), but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, the growth of microorganisms on hydrocarbons presents a special problem, since not only are hydrocarbons immiscible in water, but also their breakdown cannot occur extracellularly. The first step in aromatic (6) or aliphatic (19) hydrocarbon degradation is the introduction of molecular oxygen into the molecules by cell-associated enzymes (5).

Two general types of hydrocarbon-cell interactions, depending upon the state and size of the oil droplets relative to the size of the microbial cells, have been postulated (9): the adherence of cells to large oil droplets and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets. The relative contribution of each of these two types of interactions to the growth of bacteria on hydrocarbons has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon-aqueous interfacial area and growth rate can be used to support either hypothesis (16).

Hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 has been shown both to adhere avidly to hydrocarbons and other surfaces possessing hydrophobic properties (23-26), and to produce a potent extracellular emulsifying agent (20). The purified emulsifying agent, referred to as emulsan, is an anionic heteropolysaccharide (37) containing fatty acid side chains (1), with an average molecular weight of 9.9 × 10^5 and an axial ratio of about 60. In this paper, we describe the isolation and characterization of a mutant of RAG-1 that is deficient in its ability to adhere to hydrocarbons. By using this mutant we have been able to demonstrate the importance of adherence in the growth of RAG-1 on hexadecane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) and a nonadhering derivative, MR-481, were used throughout this study. Cells were maintained on nutrient agar at 4°C and were subcultured every 2 months.

For measuring adherence to test hydrocarbons or for preparing inocula for growth experiments, 0.1 ml of bacteria from an overnight culture was inoculated into 50 ml of brain heart infusion broth (Difco Laboratories) and incubated in a New Brunswick model G-53 Gyrotory shaker at 150 rpm at 30°C. Cells were harvested in the stationary phase after 18 h of growth, washed twice, and suspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, distilled water to 1,000 ml [pH 7.1]).

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14-mm diameter) containing 4.2 ml of aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml of hexadecane (olefin-free, 99% purity; Fluka AG, Buchs SG, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick Gyrotory water bath shaker (model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Isolation of nonadhering mutant MR-481. A spontaneous mutant strain of RAG-1 which is deficient in its ability to adhere to hydrocarbons was isolated by the following technique. RAG-1 cells were inoculated into 50 ml of nutrient broth (Difco) supplemented with 0.5% NaCl and incubated in a New Brunswick model G-53 Gyrotory shaker at 150 rpm at 30°C. Cells were harvested in the stationary phase after 18 h of growth, washed twice, and suspended in PUM buffer to an absorbance at 400 nm of ca. 1.5 (Gilford model 240 spectrophotometer; 1-cm light path). To 5 ml of the cell suspension was added 2 ml of *n*-octane, and the phases were mixed in a Vortex mixer for 5 min. After phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases were mixed as described above. This extraction process was continued until the cells in the lower aqueous phase were too few to be visible by microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to the stationary phase as described above. Cells were again harvested, washed, and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even after several extraction attempts with octane. The final aqueous phase was plated on nutrient agar, and three individual colony types were isolated and tested for adherence to hydrocarbons. One strain, termed MR-481, exhibited no significant affinity towards the test hydrocarbons and was chosen for further study.

Adherence to hydrocarbons. The technique for measuring adherence of bacteria to hydrocarbons has been described previously (24). Cells were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of ca. 1.5. Various volumes of the test hydrocarbons (*n*-hexadecane, *n*-octane, and *p*-xylene) were added to round-bottom acid-washed test tubes (10-mm diameter) containing 1.2 ml of washed-cell suspension, and the phases were mixed in a Vortex mixer uniformly for 120 s. After 15 min was allowed for the phases to separate, the absorbance of the lower aqueous phase of each tube was measured at 400 nm. Adherence was calculated as the percent loss in absorbance relative to that of the initial cell suspension.

Emulsan preparation. The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously (22). The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing it extensively against distilled water. The material was then deproteinized by the phenol method (37) and dialyzed. The final product had a reduced viscosity of 490 cm^3/g, an emulsifying activity of 150 U/mg, and an ester content of 0.5 μmol/mg and contained less than 0.5% protein. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and sterilized by autoclaving before it was added to RAG-1 and MR-481 in the growth experiments.

RESULTS

Isolation and characteristics of *A. calcoaceticus* MR-481. *A. calcoaceticus* RAG-1 adheres avidly to hydrocarbons (24–26). Adherent cells were observed exclusively on the surface, rather than in the interior, of the hydrocarbon droplets. A spontaneous mutant strain of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells which did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when it was grown under conditions for which RAG-1 adherence was optimal (Fig. 1). Upon being mixed, RAG-1 broke the hydrocarbon phase into small droplets that were covered by adherent cells. These droplets rose rapidly after the mixing procedure of the assay; a dramatic decrease in the absorbance of the lower aqueous phase, corresponding to removal of adhering cells, was observed. Mutant MR-481, however, was nonadherent, and the hydrocarbon phase rapidly coalesced after being mixed, leaving the cells in the aqueous phase.

Mutant MR-481 resembled the wild type in the following characteristics: colonial and cell morphology; sensitivity to two bacteriophages, AP-2 and AP-3 (O. Pines and D. L. Gutnick, Arch. Microbiol., in press), which are specific for *A. calcoaceticus* RAG-1 strains; production of extracellular emulsifying activity during growth on ethanol as the sole carbon and energy source (22); and growth rate on brain heart infusion broth (38 min at 30°C with aeration).

Growth of RAG-1 and MR-481 on hexa-

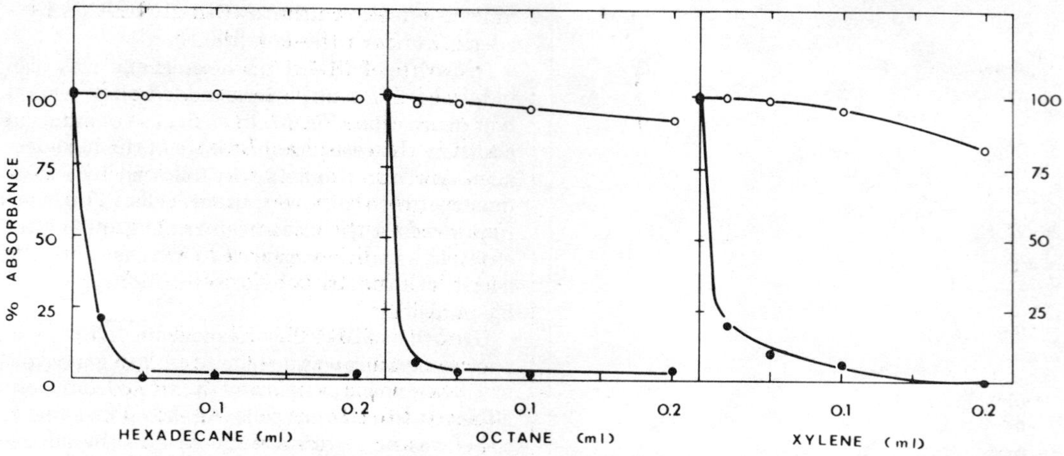

FIG. 1. *Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer to an absorbance at 400 nm of 1.45 to 1.50. To 1.2 ml of suspended RAG-1* (●) *or MR-481* (○) *cells were added various volumes of test hydrocarbon, and the mixtures were mixed in a Vortex mixer for 120 s. After phase separation, the turbidity of the lower aqueous phase was measured. The ordinate is the percentage of initial absorbance of the cell suspension.*

decane. It should be emphasized that in all growth experiments inocula were prepared in the same manner for measuring adherence as was described in the legend to Fig. 1. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 2). After a 2-h lag, both strains grew with a doubling time of 48 min.

With hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew with no appreciable lag, whereas the nonadherent mutant MR-481 failed to grow for at least 54 h (Fig. 3). The growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered with patches of adhering cells. Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus, the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for the growth of RAG-1 on hexadecane should be considered minimal, since attached bacteria were not counted. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Fig. 4).

Effect of emulsan on growth of MR-481 on hexadecane. *A. calcoaceticus* RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan. It was of interest to examine the effect of a highly purified preparation of emulsan (see above) on the growth of RAG-1 and of the nonadherent mutant MR-481 on hexadecane (Fig. 5). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481. However, emulsan did permit the growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both emulsan and hexadecane began after about 6 h, whereas neither component alone supported growth for 54 h. At the weight

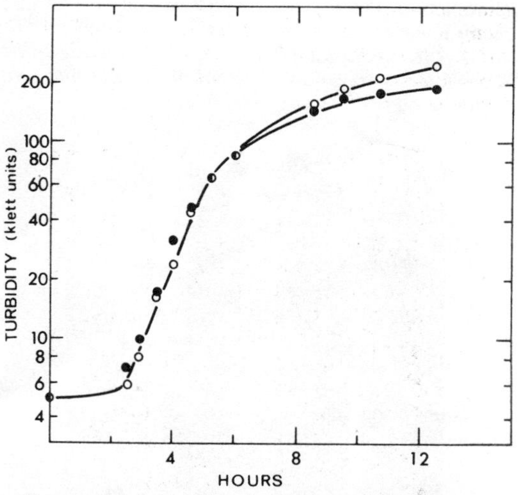

FIG. 2. *Growth of RAG-1 and MR-481 in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer supplemented with 0.2% sodium acetate. RAG-1* (●) *and MR-481* (○) *cells were inoculated into 4.2 ml of acetate medium in Klett tubes to an initial turbidity of 5 Klett units.*

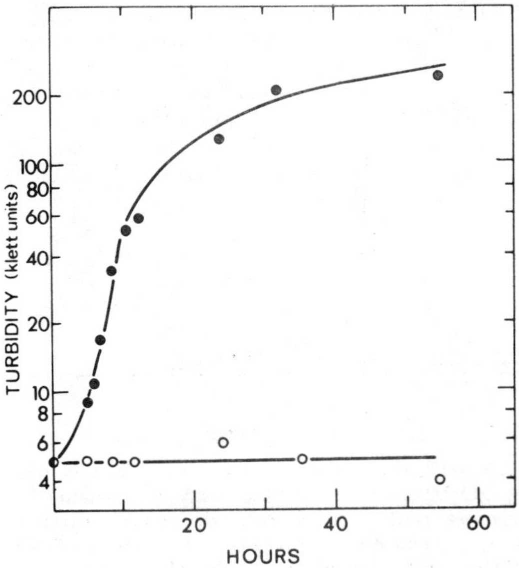

FIG. 3. *Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer were inoculated with either RAG-1 (●) or MR-481 (○) to an initial turbidity of 5 Klett units. The tubes were overlaid with 0.5 ml of hexadecane and incubated as described in the text.*

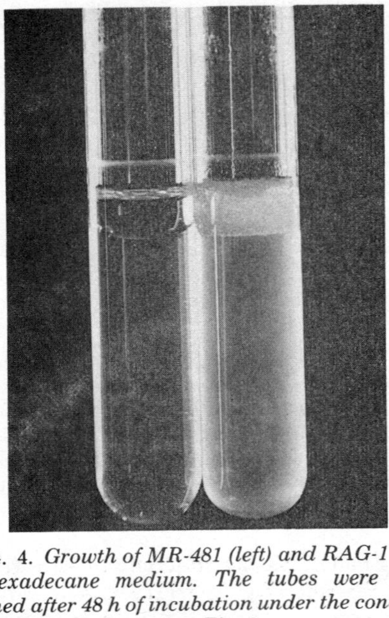

FIG. 4. *Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. The tubes were photographed after 48 h of incubation under the conditions described in the legend to Fig. 3.*

ratio of hexadecane to emulsan used, 1,925:1, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements (Z. Zosim, unpublished data). Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several h of incubation (Fig. 6). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth after emulsan addition appeared to increase with the length of incubation before the addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of a genotypic or phenotypic reversion of MR-481 from nonadherent to adherent cells. As shown in Table 1, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared with that of the initial inoculum grown in rich medium (Fig. 1).

RAG-1 cells which were harvested and washed after growth on hexadecane both in the presence and in the absence of emulsan exhibited a high affinity for hexadecane (Table 1). Thus, the appearance of free RAG-1 cells in the

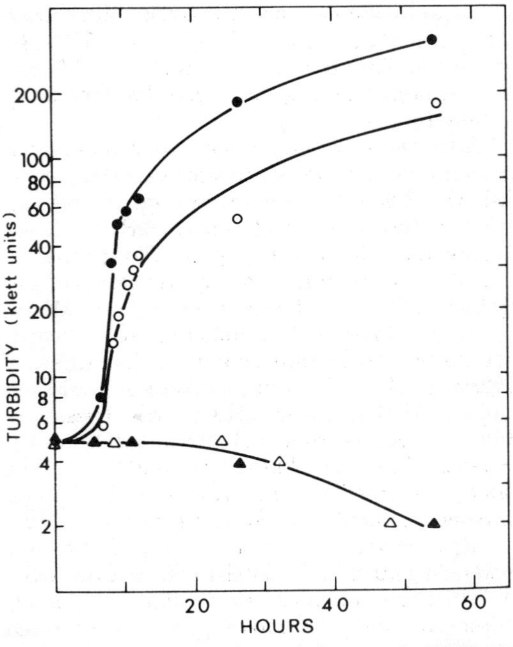

FIG. 5. *Effect of emulsan on the growth of MR-481 on hexadecane. The preparation of inocula and incubation conditions were as described in the legend to Fig. 3. RAG-1 (●) and MR-481 (○) were grown on hexadecane medium in the presence of 50 μg of emulsan per ml. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg of emulsan per ml in the absence of hexadecane.*

lower phase during growth on hexadecane did not result from a loss of their adherent properties.

DISCUSSION

Mudd and Mudd (15) reported over 50 years ago that certain microorganisms tend to concentrate at oil-water interfaces. Although this observation has been frequently corroborated during studies on the growth of microorganisms on liquid hydrocarbons (7, 9, 13, 14, 16, 17), there is no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Recent studies have shown that certain bacterial species can adhere to liquid hydrocarbons despite their inability to degrade paraffins (24) and that *Pseudomonas aeruginosa* cells exhibit a low affinity for hydrocarbons, even when they are grown on hexadecane (26). Certain investigators have argued that microbial growth on hydrocarbons is caused by emulsification of the water-insoluble substrate rather than by direct contact (2, 34, 35). *A. calcoaceticus* RAG-1 is an interesting microorganism in this regard because it both adheres avidly to hydrocarbons (24–26) and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized (1, 20, 22, 37).

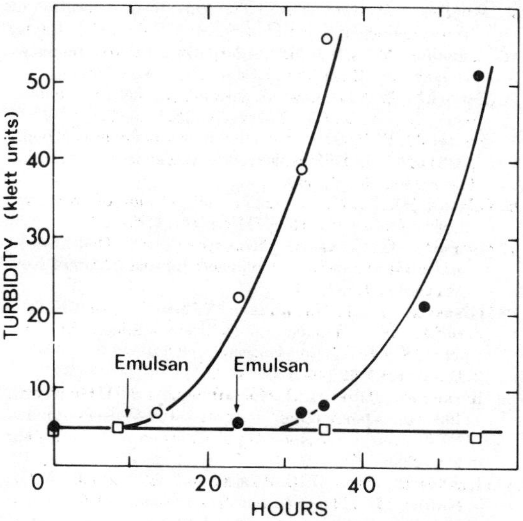

FIG. 6. *Induction of growth of MR-481 on hexadecane by emulsan addition. The preparation of inocula and incubation conditions were as described in the legend to Fig. 3. Emulsan was added to a final concentration of 50 μg/ml at 10 (○) or 24 h (●). Tubes containing no emulsan served as controls (□).*

TABLE 1. *Adherence of RAG-1 and MR-481 after growth on hexadecane*

Strain	Turbidity[a]		Adherence (%)
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR 481[c]	1.43	1.36	4.9

[a] Cells were washed twice and suspended in PUM buffer to initial turbidities (absorbance at 400 nm) as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension, and the mixture was mixed in a Vortex mixer for 120 s. After phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested after 54 h of growth on hexadecane medium, washed twice, and suspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested after 54 h of growth on hexadecane medium in the presence of 50 μg of emulsan per ml, washed twice, and suspended in PUM buffer.

The isolation of a mutant strain of *A. calcoaceticus* RAG-1 that is deficient in its ability to adhere to hydrocarbon permitted an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The nonadherent mutant strain MR-481 failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild-type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were ruled out by the following data: (i) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (ii) MR-481 was able to grow on hexadecane medium after the addition of an emulsifier; and (iii) MR-481 cells were able to grow on solid medium supplemented with hexadecane vapors as the sole carbon and energy source. We conclude from these data that adherence is a prerequisite for the growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of the substrate.

Both RAG-1 and MR-481 produced a potent extracellular emulsifying agent which permitted growth of nonadhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell density-dependent phenomenon (10, 21). With the low cell density of the inoculum employed (5×10^7 cells per ml), the concentration of emulsan which the cells produced was too low to emulsify the hydrocarbon and permit growth.

These experiments suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. Such conditions prevail in most natural environments. On the other hand, the presence of extracellular emulsifying agents and the provision of vigorous agitation permit the growth of nonadherent bacteria on hydrocarbons. However, such conditions, which are dependent on a diffusible substance, may be restricted to growth on hydrocarbon in closed systems such as laboratory flasks and fermentors.

The results presented here suggest that the choice of microbial strains and growth conditions largely dictates which of the two mechanisms (adherence or emulsification) will predominate in any given investigation. Similar conclusions were drawn by Nakahara et al. (16) based on the kinetics of growth of cells in fermentors. For example, models based on investigations which employed poorly adhering *Pseudomonas* strains and which were carried out in the presence of extraneous emulsifiers or with vigorous agitation (2, 8, 34, 35) tend to minimize the role of adherence in hydrocarbon degradation. In an investigation which employed conditions which prevented the emulsification of the substrate (14), direct contact was reported to be the more important factor.

An interesting phenomenon which has also been observed by other investigators during growth of *A. calcoaceticus* on hexadecane is the appearance of free cells in the aqueous phase. Neufeld et al. (17) reported that the appearance of unbound bacteria coincides with a decrease in the surface hydrophobicity of the cells. In the present study, no evidence was found that the cells which appeared in the aqueous phase during growth had lost their ability to adhere to hydrocarbon. RAG-1 cells which were harvested from the aqueous phase and washed after 54 h of growth still adhered avidly to hexadecane. The appearance of unbound RAG-1 cells in this study can best be explained by emulsifier production during growth on hexadecane (22). The presence of emulsifier in hydrocarbon fermentation is accompanied by a decrease in interfacial tension between the hydrocarbon and aqueous phases (14, 17) which may, in turn, affect the thermodynamic favorability of cell partitioning at the interface.

The importance of bacterial adherence in the successful colonization of a wide variety of surfaces has gained increasing recognition in recent years (11–13, 18, 27, 32, 36). In the present report, the role of adherence in the growth of RAG-1 on hexadecane has been demonstrated. Experiments are in progress to characterize the cell surface components responsible for the adherent properties exhibited by *A. calcoaceticus* RAG-1.

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LITERATURE CITED

1. **Belsky, I., D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. **101**:175–178.
2. **Chakravarty, M., H. D. Singh, and J. N. Baruah.** 1975. A kinetic model for microbial growth on liquid hydrocarbons. Biotechnol. Bioeng. **17**:399–412.
3. **Daniels, S. L.** 1980. Mechanisms involved in sorption of microorganisms to solid surfaces, p. 7–58. *In* G. Bitton and K. C. Marshall (ed.), Adsorption of microorganisms to surfaces. John Wiley & Sons, Inc., New York.
4. **Dworkin, M.** 1973. Cell-cell interactions in the myxobacteria. Symp. Soc. Gen. Microbiol. **23**:125–142.
5. **Foster, J. W.** 1962. Bacterial oxidation of hydrocarbons, p. 241–261. *In* O. Hayaishi (ed.), Oxygenases. Academic Press, Inc., New York.
6. **Gibson, D. T.** 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. **1**:199–223.
7. **Gutnick, D. L., and E. Rosenberg.** 1977. Oil tankers and pollution: a microbiological approach. Annu. Rev. Microbiol. **31**:379–396.
8. **Hisatsuka, K., T. Nakahara, Y. Minoda, and K. Yamada.** 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agric. Biol. Chem. **39**:999–1005.
9. **Kirschner Zilber, I., E. Rosenberg, and D. Gutnick.** 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on *n*-tetracosane. Appl. Environ. Microbiol. **40**: 1086–1093.
10. **Lankford, C. E., J. R. Walker, J. B. Reeves, N. H. Nabbut, B. R. Byers, and R. J. Jones.** 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s) ("schizokinen"). J. Bacteriol. **91**:1070–1079.
11. **Latham, M. J.** 1980. Adhesion of rumen bacteria to plant cell walls, p. 339–350. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.
12. **Lippincott, J. A., and B. Lippincott.** 1980. Microbial adherence in plants, p. 375–398. *In* E. H. Beachey, (ed.), Bacterial adherence. Chapman and Hall, Ltd., London.
13. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass.
14. **McLee, A. G., and S. L. Davies.** 1972. Linear growth of a *Torulopsis* sp. on n-alkanes. Can. J. Microbiol. **18**: 315–319.
15. **Mudd, S., and E. B. H. Mudd.** 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. **40**:633–645.
16. **Nakahara, T., L. E. Erickson, and J. R. Gutierrez.** 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol. Bioeng. **19**:9–25.
17. **Neufeld, R. J., J. E. Zajic, and D. F. Gerson.** 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. Appl. Environ. Microbiol. **39**:511–517.
18. **Ofek, I., and E. H. Beachey.** 1980. General concepts and principles of bacterial adherence in animals and man, p. 1–31. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, Ltd., London.
19. **Ratledge, C.** 1978. Degradation of aliphatic hydrocarbons, p. 1–46. *In* R. J. Watkinson (ed.), Developments in biodegradation of hydrocarbons, vol. I. Applied Science Publishers, London.
20. **Reisfeld, A., E. Rosenberg, and D. Gutnick.** 1972.

Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Environ. Microbiol. **24**:363-368.

21. **Rosenberg, E., K. H. Keller, and M. Dworkin.** 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. J. Bacteriol. **129**:770-777.
22. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402-408.
23. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42**:375-377.
24. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29-33.
25. **Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg, and I. Ofek.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. **33**:29-33.
26. **Rosenberg, M., E. Rosenberg, and D. Gutnick.** 1980. Bacterial adherence to hydrocarbons, p. 541-542. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.) Microbial adhesion to surfaces. Society of Chemical Industry, London.
27. **Schaeffer, W. I., P. E. Holbert, and W. W. Umbreit.** 1963. Attachment of *Thiobacillus thiooxidans* to sulfur crystals. J. Bacteriol. **85**:137-140.
28. **Shapiro, L.** 1976. Differentiation in the caulobacter cell cycle. Annu. Rev. Microbiol. **30**:377-407.
29. **Stanier, R. Y.** 1942. The cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. **6**:143-196.
30. **Starr, M. P., and J. C. C. Huang.** 1972. Physiology of the *Bdellovibrios*, p. 21-261. *In* A. H. Rose and D. W. Tempest (ed.), Advances of microbiological physiology, vol. 8. Academic Press, Inc., London.
31. **Sundarrj, C. P., and J. V. Bhat.** 1972. Breakdown of chitin by *Cytophaga johnsoni*. Arch. Mikrobiol. **85**: 159-167.
32. **Takakuwa, S., T. Funimori, and H. Iwasaki.** 1979. Some properties of cell-sulfur adhesion in *T. thiooxidans*. J. Gen. Appl. Microbiol. **25**:21-29.
33. **Varon, M., and M. Shilo.** 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *Bdellovibrio bacteriovorus*. J. Bacteriol. **95**:744-753.
34. **Velankar, S. K., S. M. Barnett, C. W. Houston, and A. R. Thompson.** 1975. Microbial growth on hydrocarbons—some experimental results. Biotechnol. Bioeng. **17**:241-251.
35. **Wang, D. I. C., and A. Ochoa.** 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentations and relationships to specific growth rates. Biotechnol. Bioeng. **14**:345-360.
36. **Weiss, R. L.** 1973 Attachment of bacteria to sulphur in extreme environments. J. Gen. Microbiol. **77**:501-507.
37. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties Appl. Environ. Microbiol. **37**:414-420.

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Bacterial Adherence to Polystyrene: a Replica Method of Screening for Bacterial Hydrophobicity

MEL ROSENBERG

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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A simple replica method is described for the rapid identification of colonies of bacteria which adhere to polystyrene. A correlation was found between the adherence of bacterial strains to polystyrene and cell surface hydrophobicity, suggesting the use of this technique in screening for cell surface mutants and in the isolation of hydrophobic bacteria from nature.

Hydrophobic interactions play a role in the adherence of microorganisms to a wide variety of surfaces. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in the partitioning of microorganisms at interfaces (2, 8), in the adherence of bacteria to nonwettable plastic surfaces (3, 5, 6), in the attachment of bacteria to phagocytes (15) and other mammalian cells (1, 9, 10a, 12), and in the growth of cells on insoluble hydrophobic substrates, such as hydrocarbons (M. Rosenberg and E. Rosenberg, J. Bacteriol., in press).

We previously described a rapid method for the measurement of bacterial surface hydrophobicity based on the adherence of washed cells to liquid hydrocarbons (10, 11). This technique, as well as other methods for measuring bacterial hydrophobicity (7, 9, 12, 13), is inadequate for the simultaneous screening of large numbers of bacterial samples.

In this report a simple method for the rapid initial identification of colonies of hydrophobic cells is described. This technique is based on differential cell adherence to polystyrene. The adherence of a number of bacterial species to polystyrene has been investigated by several authors and attributed to hydrophobic interactions between cells and the plastic surface (5, 6). In preliminary experiments we found a direct correlation between bacterial strains which showed a high affinity for liquid hydrocarbons and the ability of these strains to adhere to polystyrene. Similarly, bacterial strains which did not adhere to hydrocarbons could easily be displaced from the polystyrene surface by washing. These results suggested that a simple replica plate method could be used as a rapid test for the screening of bacterial colonies for cell surface hydrophobicity.

In the proposed technique, a flat polystyrene disk is pressed onto the surface of an agar plate containing the colonies to be screened. Two types of sterile disks were employed: (i) 25-mm-diameter, untreated, round polystyrene cover slips supplied by Lux Scientific Corp., Newbury Park, Calif.; (ii) 80-mm-diameter disks cut from standard polystyrene petri dishes (Miniplast, Ein Shemer, Israel; and NUNC, Roskilde, Denmark). The replica of the colonies obtained on the polystyrene surface was then washed for 2 min under a vigorous stream of tap water to remove all cells which were not firmly bound. At this stage, translucent areas corresponding to colonies of adherent cells could be observed on the polystyrene surface; microscopic observation of these spots revealed a dense layer of attached cells. To facilitate visualization and comparison with the original colonies, the replica was fixed by dipping in methanol and stained with gentian violet.

Results obtained by this technique are shown in Fig. 1 to 4. In all of the experiments, cells were grown on nutrient agar plates supplemented with 0.5% NaCl and examined after 48 h of incubation. In Fig. 1, colonies of *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012), a strain which adheres avidly to hydrocarbons (10, 11), were compared with colonies of MR-481, a mutant derived from RAG-1 which is deficient in cell surface hydrophobicity and unable to adhere to hydrocarbons (10a; Rosenberg and Rosenberg, J. Bacteriol., in press). Mutant MR-481 cells were completely removed from the polystyrene replica in the washing procedure, whereas a clear replica of RAG-1 colonies was obtained. Mixtures of RAG-1 and MR-481 colonies which were not distinguishable by colony morphology could be easily resolved by this method (Fig. 2).

Most laboratory strains tested did not adhere to polystyrene and were removed from the disks after the washing procedure. These included colonies of *Escherichia coli* K-12 DR107, *Micro-*

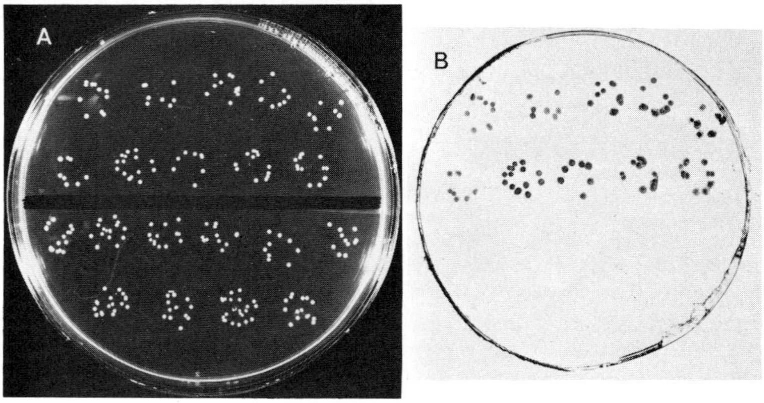

FIG. 1. *Replica of RAG-1 and MR-481 colonies. A nutrient agar plate (A) containing colonies of RAG-1 (upper half) and MR-481 (lower half) was replicated onto polystyrene, washed, and stained as described in the text. Only RAG-1 cells were adherent (B).*

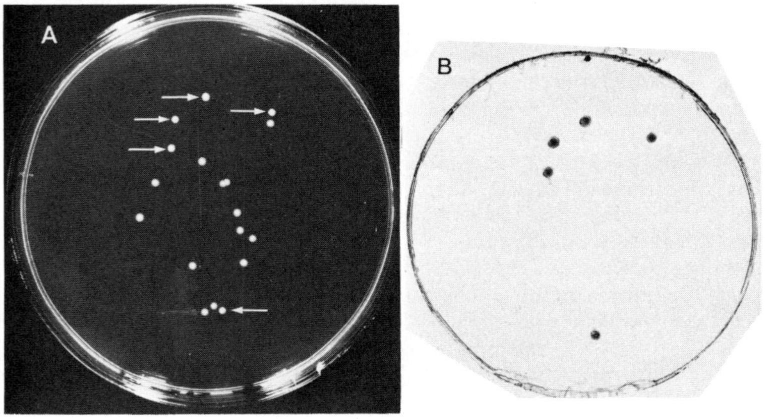

FIG. 2. *Replica of mixed RAG-1 and MR-481 colonies. A mixture of MR-481 and RAG-1 cells was plated onto a nutrient agar plate (A). On the polystyrene replica (B), five colonies of adherent cells, indicated by arrows (A), were identified.*

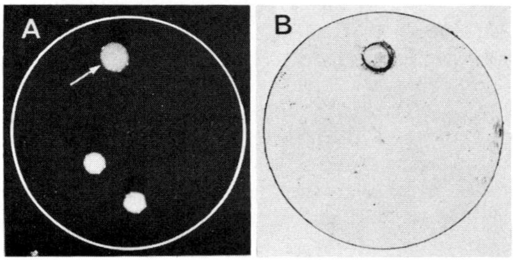

FIG. 3. *Comparison of S. aureus and S. albus colonies. A colony of S. aureus (A, arrow) and two adjacent colonies of S. albus were replicated onto polystyrene (B). A ring of stained cells, corresponding to the S. aureus colony, was observed on the replica, whereas nonadherent S. albus cells were removed during the washing procedure.*

coccus lysodeikticus (ATCC 4698), *Myxococcus xanthus* FB, *Pseudomonas aeruginosa* PAS279, *A. calcoaceticus* (ATCC 17903), and strains of *Photobacterium fischeri*, *Photobacterium leiognathi*, and *Photobacterium phosphoreum*.

Adherence of *Serratia marcescens* and *Serratia liquefaciens* strains to polystyrene was found to depend on the age of the colonies. Whereas pink- and red-pigmented colonies were replicated onto the polystyrene, young nonpigmented colonies were not adherent. The appearance of pigment in *Serratia* cells has been correlated with increasing cell surface hydrophobicity (2, 7, 10).

The stained replica of adjacent colonies of *Staphylococcus albus* and *Staphylococcus aureus* (10) is shown in Fig. 3. A characteristic ring

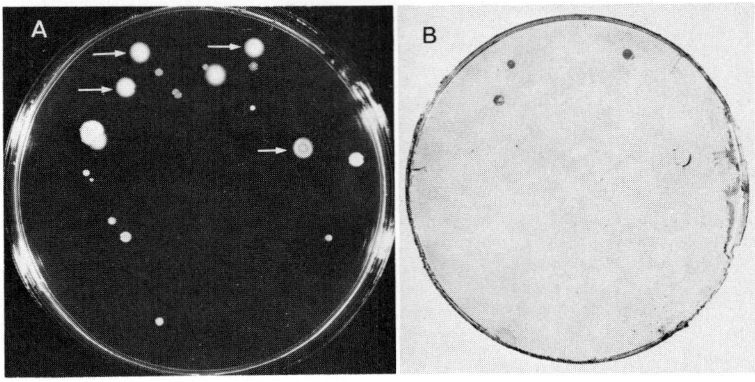

FIG. 4. *Replica of colonies from a soil sample. A sample of soil was diluted and plated onto nutrient agar (A) and incubated at 30°C for 48 h. Several adherent colonies (arrows) were identified on the polystyrene replica (B).*

of stained adherent *S. aureus* cells was obtained, whereas *S. albus* cells were completely washed from the polystyrene surface. The appearance of a ring of adherent cells, rather than a homogeneous spot, might indicate the interference by a cell-free component which coated the polystyrene at the point of contact of the colony and the disk and prevented the adherence of cells to the "conditioned" (4) surface. In a prior study of these two strains, only *S. aureus* cells exhibited affinity toward hydrocarbon (10).

Colonies of adherent cells could readily be identified in isolates from natural sources. For example, the replication of a plate of soil microorganisms yielded several adherent colonies (Fig. 4).

Results presented here further demonstrate the strong correlation between cell surface hydrophobicity and the affinity of bacteria for polystyrene. The main advantage of the described technique is that large numbers of bacterial colonies can be examined simultaneously using inexpensive and readily obtainable materials. The simplicity of this method suggests its implementation in screening for mutants in cell surface hydrophobicity and in the isolation of hydrophobic microorganisms from natural sources.

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LITERATURE CITED

1. **Beachey, E. H., W. A. Simpson, and I. Ofek.** 1980. Interaction of surface polymers of *Streptococcus pyogenes* with animal cells, p. 389–405. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.
2. **Blanchard, D. C., and L. D. Syzdek.** 1978. Seven problems in bubble and jet drop researches. Limnol. Oceangr. **23**:389–400.
3. **Dexter, S. C., J. D. Sullivan, Jr., J. Williams III, and S. W. Watson.** 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl. Microbiol. **30**:298–308.
4. **Fletcher, M.** 1976. The effects of proteins on bacterial attachment to polystyrene. J. Gen. Microbiol. **94**:400–404.
5. **Fletcher, M., and G. I. Loeb.** 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. **37**:67–72.
6. **Gerson, D. F., and D. Scheer.** 1980. Cell surface energy, contact angles and phase partition. III. Adhesion of bacterial cells to hydrophobic surfaces. Biochim. Biophys. Acta **602**:506–510.
7. **Kjelleberg, S., C. Lagercrantz, and T. Larsson.** 1980. Quantitative analysis of bacterial hydrophobicity studies by the binding of dodecanoic acid. FEMS Microbiol. Lett. **7**:41–44.
8. **Marshall, K. D.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass.
9. **Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tagesson.** 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Pathol. Microbiol. Scand. Sect. B **85**:308–316.
10. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEBS Microbiol. Lett. **9**:29–33.

10a. **Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg, and I. Ofek.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. **33**:29–33.

11. **Rosenberg, M., E. Rosenberg, and D. Gutnick.** 1980. Bacterial adherence to hydrocarbons, p. 541–542. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.
12. **Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22**:462–472.
13. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32**:19–39.

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Cell surface hydrophobicity of smooth and rough *Proteus mirabilis* strains as determined by adherence to hydrocarbons

Mel Rosenberg, Shlomo Rottem * and Eugene Rosenberg

*Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, and * Department of Membrane and Ultrastructure Research, The Hebrew University–Hadassah Medical School, Jerusalem, Israel*

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INTRODUCTION

The ability of bacteria to adhere to surfaces is recognized as an essential step in the colonization of mammalian tissues [1] as well as numerous other ecological niches [2]. An important factor in determining bacterial adherence is the hydrophobic nature of the bacterial cell surface [3]. Hydrophobic interactions have been implicated in adherence of bacteria to phagocytes [4–6], host tissues [1,6–9], non-wettable plastics [10,11], hydrocarbons [12,13], and in the partitioning of bacteria at the water : air interface [14].

Rough mutants of various Gram-negative bacteria have been shown to differ in their adherent properties as compared to the corresponding smooth parental strains [5,6,8,15]. In several cases it was suggested that the increased adherence of rough strains might be due to increased surface hydrophobicity of these strains compared to the wild type smooth strains [5,6,16,17]. We recently described a simple method for measuring bacterial surface hydrophobicity based on the adherence of cells to liquid hydrocarbons [12,13].

In the present study we demonstrate that rough mutants of *Proteus mirabilis* have a higher surface hydrophobicity than the smooth S1959 strain. The rationale for the differences in hydrophobicity and the possibility that these differences are associated with distinct adherence properties of these organisms [15] are discussed.

2. MATERIALS AND METHODS

The smooth *P. mirabilis* strains S1959 and its rough mutants R45 and R51 were grown at 37°C with vigorous shaking in nutrient broth supplemented with 1.2% NaCl. Cells were harvested in stationary phase, following 20 h growth, washed twice and suspended in PUM buffer, pH 7.1: 22.2 g $K_2HPO_4 \cdot 3\,H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7\,H_2O$ and distilled water to 1000 ml. The light absorbance of the cell suspension at 400 nm was adjusted to 1.4–1.5 using a Gilford Model 240 spectrophotometer. Adherence to the three test hydrocarbons (*n*-hexadecane, *n*-octane and *p*-xylene) was assayed as described previously [12,13]. In brief, acid-washed test tubes containing 1.2 ml of the cell suspension were vortexed on a Thermolyne Maxi Mix (Sybron) in the presence of different volumes of test hydrocarbon for 120 s. After phase separation, the light absorbance of the lower aqueous phase was measured at 400 nm and compared with that of the cell suspension prior to the mixing procedure.

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3. RESULTS AND DISCUSSION

The adherence of *P. mirabilis* strains S1959, R51 and R45 is shown in Fig. 1. A decrease in the percent absorbance indicates the removal of cells from the bulk aqueous phase to the aqueous:hydrocarbon interface. Smooth strain S1959 exhibited no significant affinity towards any of the test hydrocarbons. Rough strain R51 was unable to adhere to hexadecane or octane, but did show a slight affinity towards xylene. Deep rough strain R45 showed a slight affinity towards hexadecane and octane and a pronounced affinity towards xylene. Microscopic observation of the xylene droplets revealed R45 cells adhering in a layer at the hydrocarbon surface. Age of the culture did not significantly affect the adherence of the cells to hydrocarbons, as similar affinities to those shown in Fig. 1 were observed with cells harvested during exponential growth phase.

Results presented here agree with studies on other bacterial strains which have reported that rough strains exhibit greater cell surface hydrophobicity than the corresponding smooth strains [5,6,16,17]. Of the three test hydrocarbons employed, the most pronounced differences were obtained with xylene. The differences in surface hydrophobicity between the smooth and 'deep rough' *P. mirabilis* strains may be due to differences in the localization of phospholipids in the outer and inner leaflets of the outer membrane (OM) of these strains. As was suggested in studies with *Salmonella typhimurium* [18], the localization of phospholipids in the wild-type smooth strain differs from that observed in 'deep rough' mutants. In the smooth strain most of the phospholipids are present in the inner leaflet, unexposed to the surrounding medium, whereas in the deep rough mutants (i.e., Rd and Re strains) phospholipids are present in both the outer and inner leaflets of the OM. If, however, the distribution of phospholipids between the inner and outer leaflets is similar in the smooth strain and in its 'deep rough' mutants, with phospholipids present in both leaflets [19,20], the inaccessibility of the smooth strain of *P. mirabilis* to the hydrophobic surfaces studied here may be due to the shielding effect by the long hydrophilic carbohydrate chains of the lipopolysaccharide molecules of the smooth strain [21].

The use of bacterial adherence to liquid hydrocarbons as a criterion for cell surface hydrophobicity has certain advantages: elaborate equipment is not necessary and quantitative results are simply and rapidly obtained; moreover, since hydrophobic interactions have been implicated in the ability of certain bacteria to adhere to mammalian cells [1,5–9], adherence to liquid hydrocarbons provides a defined surface for adherence which

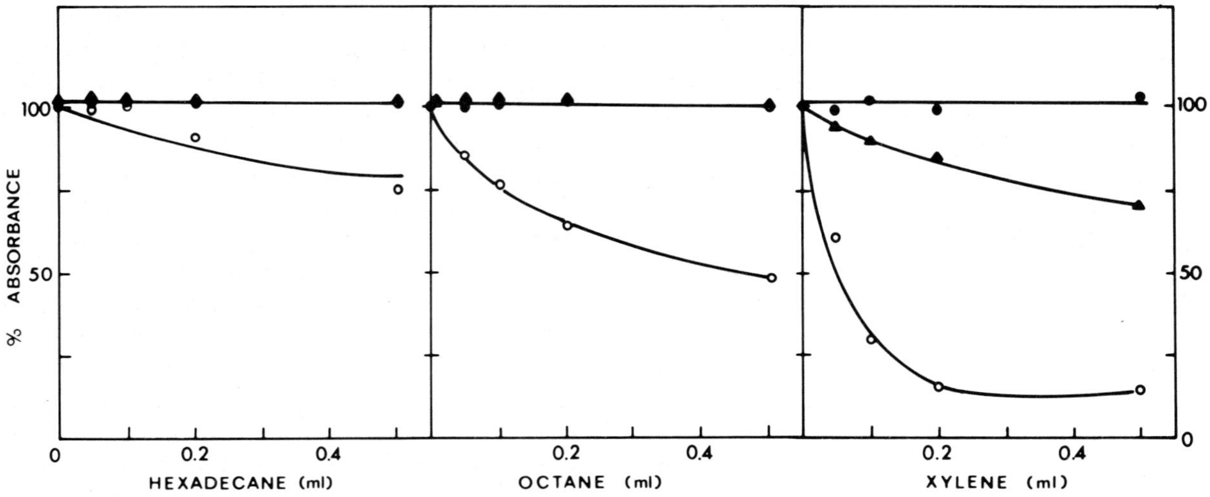

Fig. 1. Adherence of *P. mirabilis* S1959 (●), R51 (▲) and R45 (○) to hydrocarbons.

may then be compared with attachment to the less-defined biological surface. In this respect, it is of interest to note that while several studies have indicated that rough strains adhere better to phagocytes and mucosal cells than the smooth parental strains [5,6,16], a previous study employing the same *P. mirabilis* strains examined here reported increased adherence of S1959 to epithelial cells as compared to rough mutants R45 and R51 [15]. The impaired adherence ability of the rough strains was attributed to their low degree of pilation with respect to the smooth parent [15]. Since S1959 exhibited no affinity for hydrocarbons (see Fig. 1), it may be inferred that surface hydrophobicity is not a dominant factor in adherence of *P. mirabilis* S1959 to epithelial cells.

REFERENCES

[1] Ofek, I. and E.H. Beachey (1980) In: Bacterial Adherence (E.H. Beachey, Ed.). pp. 1–31, Chapman and Hall, London.

[2] Marshall, K.C. (1976) Interfaces in Microbial Ecology. Harvard University Press, Cambridge, MA.

[3] Pethica, B.A. (1980) In: Microbial Adhesion to Surfaces (R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent, Eds.). pp. 19–45, Ellis Horwood, Chichester, England.

[4] Van Oss, C.J. (1978) Annu. Rev. Microbiol. 32, 19–40.

[5] Stendahl, O., Tagesson, C. and Edebo, M. (1973) Infect. Immun. 8, 36–41.

[6] Edebo, L., Kihlström, E., Magnusson, K.-E. and Stendahl, O. (1980) In: Cell Adhesion and Motility (A.S.G. Curtis and J.D. Pitts, Eds.). pp. 65–103, Cambridge University Press, New York.

[7] Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) Infect. Immun. 33, 29–33.

[8] Perers, L., Andåker, L., Edebo, O., Stendahl, O. and Tagesson, C. (1977) Acta Pathol. Microbiol. Scand. Sect. B 85, 308–316.

[9] Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, T. (1978) Infect. Immun. 22, 462–472.

[10] Gerson, D.F. and Scheer, D. (1980) Biochim. Biophys. Acta 602, 281–284.

[11] Rosenberg, M. (1981) Appl. Environ. Microbiol. 42, 375–377.

[12] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[13] Rosenberg, M., Rosenberg, E. and Gutnick, D. (1980) In: Microbial Adhesion to Surfaces (R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent, Eds.). pp. 541–542, Ellis Horwood, Chichester, England.

[14] Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. (1981) Arch. Microbiol. 128, 267–270.

[15] Rottem, S. and Ofek, I. (1978) FEMS Microbiol. Lett. 4, 229–232.

[16] Magnusson, K.-E. and Johansson, G. (1977) FEMS Microbiol. Lett. 2, 225–228.

[17] Faris, A., Wadström, T. and Freer, J.H. (1981) Curr. Microbiol. 5, 67–72.

[18] Nikaido, H. and Nakae, T. (1979) Adv. Microbial Physiol. 20, 163–250.

[19] Verkleij, A.J., Van Alphen, L., Bijvelt, J. and Lugtenberg, B. (1977) Biochim. Biophys. Acta 466, 269–282.

[20] Rottem, S. and Leive, L. (1977) J. Biol. Chem. 252, 2077–2081.

[21] Hasin, M., Razin, S. and Rottem, S. (1976) Biochim. Biophys. Acta 433, 229–239.

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Edited by

Dr. Erle C. Donaldson, *Chairman*
Bartlesville Energy Technology Center
U.S. Department of Energy
P.O. Box 1398
Bartlesville, Oklahoma 74005

and

Dr. J. Bennett Clark, *Co-Chairman*
Phillips Petroleum Company
Research and Development
Bartlesville, Oklahoma 74004

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Microbes and Their Metabolites

Numerous microbial cultures (pure and mixed) are capable of synthesizing a variety of biochemical products from crude petroleum and its distillate fractions. The range of metabolic products from microbial consumption of petroleum is very broad, depending on environmental conditions (pressure, temperature, salinity, pH and the presence or absence of oxygen), supporting nutrients available for cell metabolism (nitrogen, phosphorus, minerals, etc.), and the specific bacteria interacting with the petroleum.

In very general terms the metabolic products may be gases (methane, hydrogen, carbon dioxide, hydrogen sulfide), low molecular weight carboxylic acids (formic, acetic, propionic, valeric), solvents (alcohols, aldehydes, ketones), polymers (proteins, polysaccharides), surface active compounds that are generally polyanionic lipids, and many other compounds ranging from simple to very complex macromolecules.

The chemical structures of most of the high molecular weight polymers produced by microbes have not been completely elucidated because of their molecular diversity and complexity. However, the renewed emphasis on these products for possible use in petroleum recovery, processing, transportation, and storage applications should result in greater emphasis on the determination of the chemical structure and properties of the microbial products.

In MEOR research, the study of specific microorganisms, or types of microorganisms, and their metabolic products is done for one of three reasons: 1) for the surface production of various compounds which, when injected into a petroleum reservoir, will enhance oil production, 2) for the injection of cells into a reservoir for *in situ* production of metabolic products which will enhance oil recovery, or 3) for the study of the microbial ecology of the reservoir.

Slodki and Camus presented a brief history on the development of xanthan gum, which is a biopolymer produced by *Xanthomonas campestris,* that has good rheological properties for polymer floods of oil reservoirs. They then discussed the biodegradability of the biopolymer. Unsterile solutions of the gum were found to be remarkably stable, which suggested to them that xanthan-degrading microorganisms are not abundant in nature. By means of culture enrichment procedures, a pure culture of a salt-tolerant *Bacillus sp.* was isolated that produced a salt-tolerant degradative enzyme.

A more prolific mixed culture producing xanthanase was obtained by adding a *Flavobacterium sp.* to the *Bacillus* culture although the *Flavobacterium* does not, by itself, produce any xanthanase. A non-salt tolerant xanthanase was found in a second *Bacillus* species. The enzymes were partially purified and characterized. Determination of the products of the enzymatic action led them to conclude that the xanthanase preparations must be mixtures of enzymes that attack all the side chain linkages in xanthan gum.

Several papers were presented on bioemulsifiers and biosurfactants. Gutnick, Zosim, and Rosenberg reported on the interaction of emulsan with hydrocarbons. Emulsan is a high molecular weight (M.W. = 10^6), water soluble, extracellular bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1. It is a d-galactosamine-containing polyanionic polysaccharide which is initially released from the cell surface as a protein complex. Removal of the protein yields a polymer, called apoemulsan, which retains emulsifying activity. Studies on the interaction of emulsan and apoemulsan revealed several interesting properties. The value of emulsan lies in its ability to form very stable emulsions with crude oils. The authors found that the higher the molecular weight of the hydrocarbon the more effective emulsan is in the formation of stable emulsions. Furthermore, the emulsion is non-wetting (non-adhesive) to metal surfaces which suggests several uses for transportation and storage of crude oils.

The bacterial strain Rsan ver [*Pseudomonas sp.*] was reported by Guerra-Santos et al to produce surface active compounds equally well with glucose or hydrocarbon as a substrate. The behavior of the culture and surfactant production were studied in a bioreactor, in batch culture, and in continuous culture. The surface tension of surfactant from batch culture growth was considerably less than that obtained in continuous culture.

The effect of surfactant hydrophile-lipophile balance (HLB) on bitumen recovery and surface activity of *Corynebacterium fascians* was reported by Zajic et al. The highest total bitumen recovery by hexadecane-grown bacteria occurred at HLB values of 5.5 and 16. Hydrophobic values (HLB 0.5 to 11) stimulated the growth and surface activity of *C. fascians* grown on such water-soluble substrates as sucrose. HLB values of 0.5, 16, and 29 improved the emulsification properties of the sucrose-grown bacteria. Membrane filtrates of culture broths to which a surfactant had been added showed that the bacterial cells, *per se,* contribute highly to the total surface activity of the culture.

According to Rosenberg et al two general types of interaction between microorganisms and hydrocarbons have been postulated: 1) adherence of cells directly to large oil droplets, and 2) release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon:aqueous interfacial area. In the case of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1, both types of interaction are operative. It was demonstrated that adherence is a prerequisite for growth on hexadecane under two conditions: low initial cell density and limited emulsification of the substrate, which are the conditions in most natural environments. Bioemulsification, however, is a cell-density-dependent phenomenon. Adherence of microorganisms to hydrocarbons was found to be neither an exclusive property of hydrocarbon-degrading microorganisms nor is it restricted to those hydrocarbons that a microorganism can metabolize.

Wang reported on a bacterial strain which produces extracellular polysaccharide from crude oil or heavy liquid paraffin as the carbon source. This was found to be a new species and was named *Brevibacterium viscogenes.* The yields of polysaccharide were 8 gm/l from 12 percent (W/V) crude oil and 12 gm/l from 4 percent (W/V) heavy liquid paraffin. The conversion rate of added paraffin was over 40 percent. The physical properties of this polysaccharide are comparable to those of xanthan and are considerably better than partially hydrolyzed polyacrylamide or carboxymethylcellulose. When the polysaccharide was used as a driving solution, an injection volume, corresponding to 20 percent of the pore volume, enhanced oil recovery to about 9 percent of the initial reserves.

The environmental parameters of the reservoir will limit the types of microorganisms which can be used for *in situ* processes. McInerney discussed the physiological types of microorganisms that would be useful in *in situ* processes. The limiting environmental factors that would affect either injectivity of cells or their growth and metabolic activities include permeability, temperature, pressure, salinity, salt composition, pH, and the nature of the residual oil. Most reservoirs are essentially anaerobic and, since there is no well documented record of truly anaerobic utilization of hydrocarbons, an external food supply would be necessary. This could add the parameter of nutrient limitation as a mechanism for controlling growth and metabolism of injected cells. The metabolism of various types of microorganisms and the potential role of metabolic types in MEOR were discussed.

Because of the anaerobic environment in reservoirs, a potentially useful group of microorganisms for MEOR would be the *Clostridia.* E. Grula et al reported on a screening program designed to isolate species of *Clostridium* that would have useful characteristics for *in situ* processes. They looked for the following characteristics: 1) production of large amounts of gas (primarily CO_2), 2) production of large amounts of low molecular weight organic acids, 3) production of large amounts of low molecular weight organic solvents (primarily alcohols and acetone), and 4) production of large amounts of low molecular weight nonionic emulsifiers that would form oil-in-water emulsions. Techniques were devised for rapid screening, and several potentially very useful *Clostridium* strains were isolated. E. Grula et al emphasized the difficulty of isolating species of *Clostridium* that performed well metabolically in high (5.0–17.5 percent) concentrations of sodium chloride.

Knowledge of the role of microorganisms in the genesis of oil is useful in developing microbial processes for enhanced oil recovery.

The distribution of microorganisms and the rate of bacterial methanogenesis in flooded oil fields was reported by Ivanov and Belyaev. It was found that the population of microorganisms and the rate of methanogenesis increased as stratal waters were freshened. This correlated with the decrease of organic carbon and the increase of bicarbonate concentration, as well as with methane isotopic composition becoming less and the bicarbonate carbon isotopic composition becoming greater. The process of bacterial oil oxidation took place in the zone of contact between the injected fresh waters and stratal waters of oil fields. The products of aerobic oil destruction stimulated the activity of methane-producing bacteria. Methods were reported to induce a considerable stimulation of activity of the stratal microflora in oil fields. This activation of microbial processes resulted in the increase of concentration of soluble organic compounds and the content of newly-produced methane in stratal water, which could enhance oil recovery.

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Cell-Surface Hydrophobicity of Adherent Oral Bacteria

Ervin Weiss,† Mel Rosenberg,‡ Herbert Judes,† and Eugene Rosenberg‡*

† Department of Operative Dentistry, School of Dental Medicine, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

‡ Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

Abstract. Adherent bacteria were released from the surfaces of four freshly extracted teeth by mild sonic oscillation, and screened for cell-surface hydrophobicity on the basis of their ability to adhere to hexadecane. Of the 103 tooth isolates examined, 82 adhered to the test hydrocarbon. Hydrophobic bacteria could similarly be isolated from the stainless steel dental matrix bands following brief incubation in the mouth of a volunteer; 30 of 52 isolates examined adhered to hexadecane. Among those strains which adhered to hexadecane, streptococci were the most frequent type isolated. Various other morphological types were also observed, including cocci, bacilli, coryneforms, and filamentous bacteria. The high overall proportion of hydrophobic bacteria found in this study (72%) suggests that cell-surface hydrophobicity may play a role in adherence of certain oral species to the tooth surface.

Cell-surface hydrophobicity is an important factor in the adherence and subsequent proliferation of microorganisms on solid surfaces and at interfaces [8]. Hydrophobic interactions play a role in the adherence of bacteria to nonwettable plastics [10], phagocytes [17], and buccal epithelial cells [12], and in the partitioning of cells at liquid-hydrocarbon [8–14] and liquid-air [1] interfaces. Adherence of petroleum-degrading bacteria to their hydrophobic substrate is a prerequisite for growth under certain conditions [13].

While the hydrophobic properties of many laboratory strains have been tested using a variety of qualitative and quantitative approaches [6, 7,9,16,17], few studies have undertaken the screening of fresh microbial isolates for cell-surface hydrophobicity [1,2,16]. The adherent properties of bacterial isolates may change with prolonged laboratory cultivation [3]. To our knowledge, there have been no previous reports on the cell-surface hydrophobicity of bacteria adhering to the tooth surface. A quantitative technique for measuring bacterial hydrophobicity based on adherence to liquid hydrocarbons was recently described [11,14]. The simplicity of this method lends itself to the rapid screening of numerous microbial samples. In the present study we have examined the hydrophobic surface characteristics of 155 fresh microbial isolates obtained from the surfaces of extracted teeth and incubated matrices. Over 70% of these isolates exhibited pronounced cell-surface hydrophobicity, as determined by adherence to hexadecane.

Materials and Methods

Isolation of microorganisms. Adherent microorganisms were isolated from four freshly extracted teeth, obtained from four patients, and from two Tofflemire stainless steel matrix bands (Teledyne, Elk Grove Village, Illinois) cut to 0.5 cm × 0.5 cm × 0.038 mm. The latter were sterilized and subsequently incubated for 2 h within an acrylic bite plate [5] in the mouth of a healthy, low-caries volunteer.

Following removal, teeth and matrices were washed extensively with 20 ml saline in sterile Petri dishes and then transferred to test tubes containing 5 ml saline and vortexed for 2 min to remove loosely bound cells. Teeth and matrices were then transferred to wells of sterile Costar dishes (Costar, Cambridge, Massachusetts) containing 5 ml saline and exposed to sonic oscillation at 0°C for 15 s at 20 watts power in a Braun Labsonic (B. Braun Melsungen AG, Melsungen, FRG) 1510 sonifier (standard probe). Appropriate dilutions were spread onto brain heart infusion agar (Difco Laboratories, Detroit, Michigan) and incubated under aerobic conditions at 37°C. Colonies were chosen randomly from these plates for further study.

Incubation conditions. Following two passages on brain heart infusion agar, isolated colonies were inoculated into test tubes containing 5 ml brain heart infusion broth (Difco) and grown for 48 h at 37°C with slow reciprocal shaking. Cells were harvested, washed twice, and suspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4$

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* To whom reprint requests should be addressed.

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$7H_2O$ and distilled water to 1,000 ml), pH 7.1, to optical densities of 1.0 to 1.5 at 400 nm, measured in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Adherence to hexadecane. The general method for measuring bacterial adherence to liquid hydrocarbons has been described previously [11,14]. To 1.2 ml of bacterial suspension, various volumes of hexadecane were added. After preincubation at 30°C, the two phases were vortexed for 120 s on a Thermolyne mixer (Sybron Corp., Dubuque, Iowa). After the phases were allowed to separate, the aqueous phase was carefully removed and its light absorbance at 400 nm measured. The proportion of adherent cells was determined by comparing the drop in absorbance following the mixing procedure with the absorbance of the suspension prior to the assay.

Results

Table 1 describes the four teeth used in the experiments. Table 2 gives the number of adherent microorganisms released from the teeth and stainless steel matrices by sonic oscillation, as determined by colony forming units on brain heart infusion agar. The two carious teeth, A and B, yielded approximately 70 times more cells than the noncarious teeth, C and D. The number of microorganisms isolated from each of the matrices was of the same order of magnitude as the number of cells released from noncarious teeth C and D. Different colonies appearing on the plates were selected at random, cloned, and subsequently examined for their ability to adhere to hexadecane.

Figure 1 illustrates the technique, showing adherence of two of the strains as a function of increasing hexadecane volume. While adherent strain M2-14 (isolated from matrix 2) was increasingly removed from the aqueous phase by increasing volumes of hexadecane, the turbidity of the suspension of cells of D-26 (from tooth D) was not significantly affected. Following the mixing procedure, adherent cells rose with the hydrocarbon droplets to form a stable upper "cream" consisting of cell-coated droplets (Figs. 2, 3). In the case of nonadherent strains, the hydrocarbon droplets coalesced following the mixing procedure, and little or no drop in turbidity of the aqueous phase was observed. For the results presented in Table 2, cell suspensions were mixed in the presence of 0.2 ml hexadecane. Strains were scored adherent positive if more than 75% of the cells were removed to the aqueous:hydrocarbon interface following the assay procedure. With few exceptions, the isolates examined were either totally adherent (over 90%) or not at all adherent (less than 10%).

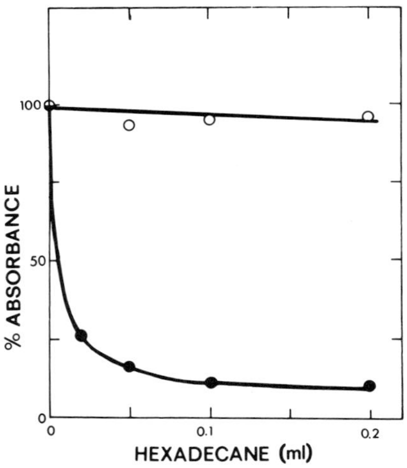

Fig. 1. Oral bacterial strains M2-14 (●) and D-26 (○) were compared for adherence to hexadecane. Initial optical absorbance: M2-14, 1.35; D-26, 1.23.

Table 1. Description of extracted teeth used for isolating adherent oral bacteria.

Tooth[a]	Condition	Reason for extraction
A	Untreated large caries	Caries
B	Untreated large caries	Caries
C	Intact	Pericorinitis
D	Root canal treatment and composite filling	Periodontitis

[a] Each of the four teeth were third molars extracted from adult males.

Table 2. Screening of oral bacteria[a] for cell surface hydrophobicity.

Source of microorganisms[a]	Total colony forming units	Isolates examined	Isolates adherent[b] to hexadecane
Tooth A	1.8×10^6	32	32
Tooth B	2.3×10^6	23	18
Tooth C	2.6×10^4	31	31
Tooth D	3.3×10^4	17	1
Matrix 1	1.2×10^4	29	13
Matrix 2	2.2×10^4	23	17

[a] Released from teeth and stainless steel matrices (see text).

[b] Isolates were designated as adherent if more than 75% of the cells were removed following mixing in the presence of 0.2 ml hexadecane.

In teeth A and C, all of the isolates examined were hydrophobic. In tooth B, 78% of the isolates adhered to hexadecane, whereas only one out of the 17 isolates from tooth D was adherent. A high frequency of hydrophobic bacteria were also isolated from the stainless steel matrices that were incubated for only two hours in a bite plate in the mouth

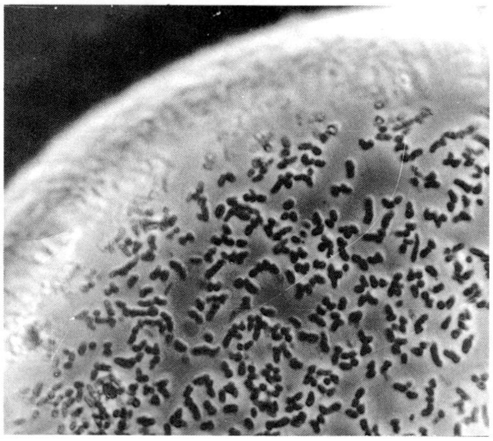

Fig. 2. Cells of oral bacterial strain M2-14 adhering to hexadecane droplet following assay procedure. ×1,200.

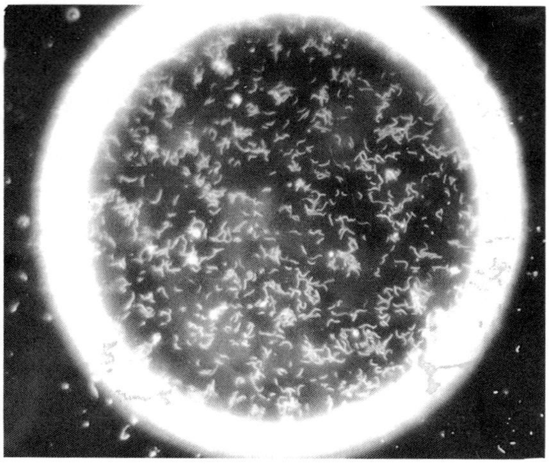

Fig. 3. Cells of the filamentous oral bacterial strain B-30 (isolated from tooth B) adhering to hexadecane droplet following assay procedure. ×200.

of a healthy volunteer (Table 2).

Although the hydrophobic bacteria isolated in the present report have not yet been classified, certain generalizations can be made: (i) All of the isolates obtained from tooth A were streptococci with similar cell and colony morphology; (ii) the single isolate from tooth D which adhered to hexadecane was a Gram-positive bacillus; (iii) hydrophobic isolates from teeth B and C and the two stainless steel matrices comprised a wide variety of morphological types, including filamentous forms, coryneforms, bacilli, streptococcal, and other coccal forms; (iv) all the streptococci examined adhered to hexadecane.

Discussion

Various studies have demonstrated that the tooth surface is normally covered by a hydrophobic film of organic matter, termed "acquired pellicle" [4]. While it has been postulated that the low surface energy imparted by this organic layer may act to impede bacterial attachment [5], it has also been suggested that hydrophobic interactions may actually participate in adherence of certain oral species to the tooth surface [3,15].

A surprisingly high overall proportion of bacteria tested (72%) exhibited pronounced ability to adhere to the test hydrocarbon. Bacterial isolates studied in this report probably represent a small fraction of the bacteria which adhere to and colonize the tooth surface, both because of the removal procedure (mild sonic oscillation) and cultivation conditions (aerobic, rich medium). Nevertheless, the diversity of adherent types found demonstrates for the first time that various species of hydrophobic bacteria can be isolated directly from the tooth surface. In addition, data obtained using the stainless steel matrices demonstrate that hydrophobic microorganisms may become attached to surfaces following even a brief incubation period in the oral cavity.

The demonstration of hydrophobic bacteria at the saliva:tooth interface raises a number of interesting questions which are now amenable to experimental examination: (i) Do hydrophobic microorganisms, similar to those isolated here, play a role in aiding or abetting formation of plaque, dental caries, and periodontal disease? (ii) Are similar mechanisms involved in adherence of these oral species to the tooth surface and to hexadecane? (iii) What intraoral factors influence the affinity of hydrophobic bacteria for the tooth:saliva interface? Further investigations into the possible role of hydrophobic interactions in mediating adherence of bacteria to the tooth surface are underway.

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Literature Cited

1. **Dahlbäck, B., Hermansson, M., Kjelleberg, S., Norkrans, B.** 1981. The hydrophobicity of bacteria—an important factor in their initial adhesion at the air-water interface. Archives of Microbiology **128:**267–270.
2. **Faris, A., Wadström, T., Freer, J. H.** 1981. Hydrophobic absorptive and hemagglutinating properties of *Escherichia coli* possessing colonization factor antigens (CFA/I or

CFA/II), type 1 pili, or other pili. Current Microbiology **5:**67–72.

3. **Gibbons, R. J.** 1980. Adhesion of bacteria to the surfaces of the mouth, pp. 351–388. In: Berkeley, R. C. W., Lynch, J. M., Melling, J., Rutter, P. R., Vincent, B. (eds.), Microbial adhesion to surfaces. Chichester, England: Ellis Horwood.
4. **Glantz, P.-O.** 1969. On wettability and adhesiveness. A study of enamel, dentine, some restorative materials, and dental plaque. Odontologisk Revy **20**(Suppl.17)**:**1–132.
5. **Glantz, P.-O.** 1977. Adhesion to teeth. International Dental Journal **27:**324–332.
6. **Kjelleberg, S., Lagercrantz, C., Larsson, Th.** 1980. Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. FEMS Microbiology Letters **7:**41–44.
7. **Magnusson, K.-E., Johansson, G.** 1977. Probing the surface of *Salmonella typhimurium* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. FEMS Microbiology Letters **2:**225–228.
8. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Cambridge, Masschusetts: Harvard University Press.
9. **Mudd, S., Mudd, E. B. H.** 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. Journal of Experimental Medicine **40:**633–645.
10. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: A replica method of screening for bacterial hydrophobicity. Applied and Environmental Microbiology **42:**375–377.
11. **Rosenberg, M., Gutnick, D., Rosenberg, E.** 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters **9:**29–33.
12. **Rosenberg, M., Perry, A., Bayer, E. A., Gutnick, D. L., Rosenberg, E., Ofek, I.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infection and Immunity **33:**29–33.
13. **Rosenberg, M., Rosenberg, E.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. Journal of Bacteriology **148:**51–57.
14. **Rosenberg, M., Rosenberg, E., Gutnick, D.** 1980. Bacterial adherence to hydrocarbons, pp. 541–542. In: Berkeley, R. C. W., Lynch, J. M., Melling, J., Rutter, P. R., Vincent, B. (eds.), Microbial adhesion to surfaces. Chichester, England: Ellis Horwood.
15. **Rutter P.** 1979. The accumulation of organisms on the teeth, pp. 139–164. In: Ellwood, D. C., Melling, J., Rutter, P. (eds.), Adhesion of microorganisms to surfaces. London: Academic Press.
16. **Smyth, C. J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S., Wadström, T.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infection and Immunity **22:**462–472.
17. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annual Review of Microbiology **32:**19–39.

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Role of Thin Fimbriae in Adherence and Growth of *Acinetobacter calcoaceticus* RAG-1 on Hexadecane

MEL ROSENBERG, EDWARD A. BAYER,† JACOB DELAREA, AND EUGENE ROSENBERG*

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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Acinetobacter calcoaceticus RAG-1, a hydrocarbon-degrading bacterium which adheres avidly to hydrocarbons and other hydrophobic surfaces, possesses numerous thin fimbriae (ca. 3.5-nm diameter) on the cell surface. MR-481, a nonadherent mutant of RAG-1 which is unable to grow on hexadecane under conditions of limited emulsification and low initial cell density, lacks these fimbriae. Prolonged incubation of MR-481 in hexadecane medium enriched for partial adherence revertants. The reappearance of thin fimbriae was observed in all such revertant strains. RAG-1 cells and partial revertant strains were agglutinated in the presence of antibody, whereas MR-481 cells were not. Another mutant, AB15, which was previously isolated on the basis of its nonagglutinability in the presence of antibody, also lacked thin fimbriae and was conditionally nonadherent. Furthermore, strain AB15 was unable to grow on hexadecane medium. Adherence of RAG-1 cells to hexadecane was considerably reduced after shearing treatment. The material removed from the cell surface by shearing of RAG-1 and the partial revertant strains yielded a single antigenic band in RAG-1 and partial revertant strains, as observed by crossed immunoelectrophoresis. This band was absent in both fimbriae-less mutants, MR-481 and AB15. The data demonstrate that the thin fimbriae of RAG-1 (i) are a major factor in adherence to polystyrene and hydrocarbon, (ii) may be crucial in enabling growth of cells on hexadecane, and (iii) constitute the major cell surface agglutinogen.

Acinetobacter calcoaceticus RAG-1 adheres avidly to hydrophobic surfaces such as liquid hydrocarbons (11, 13), polystyrene (10), and buccal epithelial cells (12). In a previous report (14), the isolation of strain MR-481, a mutant which is unable to adhere to any of these surfaces, was described. The inability of strain MR-481 to adhere to hydrocarbon was reflected in its inability to grow on hexadecane under conditions of low initial cell density and limited emulsification of the hydrocarbon substrate.

Although bacterial fimbriae have been reported to mediate a wide range of adherence phenomena (7, 15), the role of fimbriae in adherence and growth of bacteria on hydrocarbons has not been reported previously. The fimbriae of various strains of *A. calcoaceticus* were studied by Henrichsen and Blom (5). These authors found two major types of fimbriae: "thin" fimbriae, ca. 3 nm in diameter, and "thick" fimbriae, ca. 5 nm in diameter. Whereas the presence of the thick fimbriae in *Acinetobacter* strains was shown to be correlated with twitching motility, no physiological role was proposed for the thin fimbriae.

The present communication deals with the role of thin fimbriae in adherence and growth of RAG-1 on hydrocarbon. The investigation was initiated as a result of two preliminary observations: (i) nonadherent mutant MR-481 cells were not agglutinated in the presence of antibody raised against wild-type cells, and (ii) prolonged incubation of MR-481 in hexadecane medium led to the selection of partial adherence revertants. These observations prompted a comparison of the properties of RAG-1, MR-481, and partial revertant strains with those of another mutant, AB15, which was enriched on the basis of its failure to agglutinate in the presence of specific antibodies (1). The data implicate the thin fimbriae in adherence of RAG-1 to hydrophobic surfaces and in growth on hexadecane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) was originally isolated from the Mediterranean coast after enrichment on crude oil (8). Nonadherent mutant MR-481 was derived from RAG-1 (14). Strain RAG-92, a lysine auxotroph of RAG-1 (1), was kindly provided by R. Avigad. Strain AB15 is a nonagglutinating mutant derived from RAG-92 (1). Partial adherence revertant strains RV-1 and RV23-4 were derived from MR-481 as described in the Results section. Cells were maintained on nutrient agar at 4°C and were subcultured every 2 months.

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† Present address: Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel.

For measuring adherence to test hydrocarbons or for preparing inocula for growth experiments, 0.1 ml of bacteria from an overnight culture was inoculated into 50 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) in a 250-ml flask and incubated in a New Brunswick model G-53 Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 150 rpm at 30°C. Cells were harvested in the stationary phase after 18 h of growth, washed twice, and suspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, and distilled water to 1,000 ml [pH 7.1]). Alternatively, cells were grown in ethanol minimal medium: PUM buffer supplemented with 2% (vol/vol) absolute ethanol. In the case of lysine-requiring strains RAG-92 and AB15, media were supplemented with 50 μg of lysine per ml.

In the growth experiments, washed cells were inoculated into acid-washed Klett tubes (14-mm diameter) containing 4.2 ml of aqueous medium to give an initial turbidity of 5 Klett units. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter. Acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml of hexadecane (olefin-free; 99% purity; Fluka AG, Buchs SG, Switzerland) layered onto 4.2 ml of PUM buffer. Lysine (50 μg/ml) was added to acetate and hexadecane growth media in the case of lysine-requiring strains RAG-92 and AB15. The test tubes were incubated upright in a New Brunswick Gyrotory water bath shaker (model G-76) at 330 rpm at 30°C.

Bacterial adherence to hydrocarbons. The technique for measuring adherence of bacteria to hydrocarbons has been described previously (11, 13). Cells were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of ca. 1.5 (Gilford model 240 spectrophotometer; 1-cm lightpath). Various volumes of the test hydrocarbons (*n*-hexadecane, *n*-octane, and *p*-xylene) were added to acid-washed test tubes, and after 10 min of incubation at 30°C, the tubes were mixed uniformly in a Vortex mixer for 120 s. After allowing 15 min for the phases to separate, the absorbance of the lower aqueous phase was measured at 400 nm and compared with that of the bacterial suspension before the mixing procedure.

Adherence to polystyrene. A simple replica method for studying bacterial adherence to polystyrene has been described (10). In this technique, a flat polystyrene disk is pressed onto an agar plate containing the bacterial colonies to be tested. The replica formed is then extensively washed with tap water and stained with Gentian violet. Colonies of adherent cells remain bound to the polystyrene surface, whereas nonadherent cells are removed during the washing procedure.

Preparation of antibody against intact RAG-92 cells. Rabbits were injected intravenously with 10^9 washed cells of RAG-92; the bacteria were harvested from a midexponential-phase culture of RAG-92 grown on ethanol medium. The rabbits were inoculated in this manner every 3 days. Three days after the third injection, the rabbits were bled. Subsequent bleedings were performed 3 days after a booster consisting of a similar RAG-92 cell preparation. Antibody (immunoglobulin G fraction) was obtained by precipitation of antiserum in ammonium sulfate at 40% saturation at 0°C; the precipitate was suspended in phosphate-buffered saline to a concentration of ca. 2 mg of protein per ml. Protein was measured by the Lowry method (6).

Antibody-mediated agglutination. Antibody-mediated agglutination of RAG-1 cells has been described previously (1). To 0.7 ml of cell suspension in semimicrocuvettes (C. A. Greiner and Sons, Nurtingen), 0.02 ml of antibody (diluted 10-fold) was added, and the percentage decrease in absorbance as a function of time was followed at 400 nm. The absorbance of a sample containing the cell suspension, but with no antibody addition, served as the control. No decreases in absorbance with respect to the controls were observed in the presence of normal rabbit serum.

Shearing of cells. Cells were harvested after growth to stationary phase (48 h) on minimal ethanol medium, washed twice, and suspended in PUM buffer to yield a turbidity of ca. 1,000 Klett units. Cell suspensions were then subjected to shearing in a Sorval Omnimixer at a setting of 9 for 5 min at 0°C. After centrifugation to remove cells, the supernatant fluids were concentrated fivefold to a concentration of 30 to 50 μg of protein per ml by ultrafiltration with an Amicon stirred cell and PM10 membrane filter (Amicon Corp., Lexington, Mass.).

CIE. Two-dimensional (crossed) immunoelectrophoresis (CIE) of concentrated supernatant fluids of cells subjected to shearing was performed according to a modification of the technique described by Weeke (17). The stock electrophoresis buffer contained 2.06 g of sodium barbital, 0.4 g of barbituric acid, 0.1 g of sodium azide, and distilled water to 1,000 ml (pH 8.6). The buffer was diluted 1:5 in distilled water, for use both as a running buffer and as a diluent for the 1% agarose. Glass plates were coated with agarose gels (2.0 mm thick). Samples (7 μl) of the concentrated supernatant fluids of sheared cells were run in the first dimension at 25 V cm^{-1} for ca. 1.5 h (bromphenol blue was used as a marker). The second-dimension gel contained 10 μl of undiluted antibody or 10 μl of AB15-adsorbed antibody per ml of agarose gel and was run at 8 V cm^{-1} for 20 h.

The staining procedure was performed as follows. After the electrophoretic procedure, gels were pressed to minimal thickness, washed twice with 0.85% NaCl and once in distilled water, dried, and stained for 30 min in 0.02% Coomassie brilliant blue R dissolved in a solution of 7% (wt/vol) acetic acid–50% methanol. Destaining was performed with the same solution in the absence of the stain.

Electron microscopy. After growth, cells were harvested, washed, and suspended in distilled water. Cells were stained with a 0.5% solution of uranyl acetate on Formvar-coated copper grids and observed in a JEOL 100B electron microscope.

RESULTS

Isolation of partial adherence revertants RV-1 and RV23-4. The nonadherent mutant MR-481

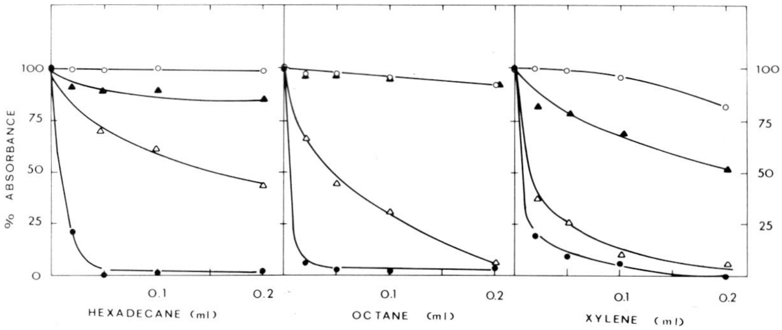

FIG. 1. Adherence of RAG-1 and derivative strains to hydrocarbon. Cells were grown to stationary phase in brain heart infusion broth. Adherence of RAG-1 (●), MR-481 (○), RV-1 (▲), and RV23-4 (△) was measured as a function of hydrocarbon volume.

failed to grow on hexadecane in liquid culture for 54 h under conditions of low initial cell density and limited emulsification of the hydrocarbon substrate (13). However, growth ensued after longer incubation periods. In one such experiment, 20 test tubes containing hexadecane growth medium were inoculated with MR-481 cells to an initial turbidity of 10 Klett units. After 72 h of incubation, considerable growth was observed in four of the test tubes (>150 Klett units). These four cultures were diluted and spread onto nutrient agar; at least 50 colonies from each culture were replicated onto polystyrene. Over 95% of the colonies tested from each of the four tubes adhered to polystyrene. In contrast, not one of 6,000 colonies obtained from the MR-481 inoculum of the same experiment adhered to the polystyrene replica. One clone was then isolated from each of the four growth tubes. These four clones were termed RV-1, RV-2, RV-3, and RV-4. In preliminary studies, the four strains appeared similar in a number of properties (adherence to hexadecane and polystyrene, agglutination by antibodies, production of emulsan after growth on ethanol [9], and cell morphology, as observed in uranyl acetate-stained electron micrographs). Strain RV-1 was arbitrarily chosen for further investigation.

To select more adherent clones, RV-1 cells were inoculated into hexadecane growth medium in test tubes and after growth were diluted

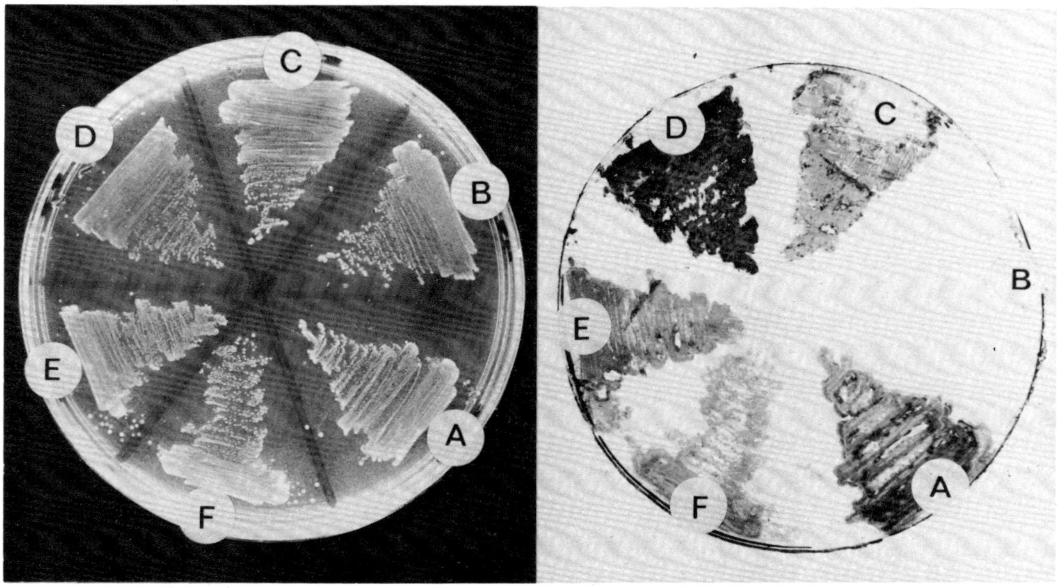

FIG. 2. Adherence of RAG-1 and derivative strains to polystyrene. Strains RAG-1 (A), MR-481 (B), RV-1 (C), RV23-4 (D), RAG-92 (E), and AB15 (F) were streaked onto a nutrient agar plate (left), and after 48 h of incubation, they were replicated onto polystyrene (right) as described in the text.

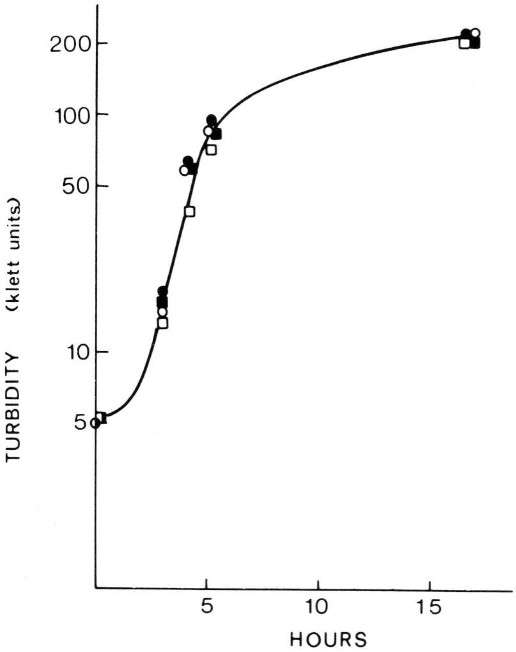

FIG. 3. Growth of RAG-1 and derivative strains in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and inoculated into 4.2 ml of acetate medium in Klett tubes. Symbols: □, RV-1; ■, RV23-4; ●, RAG-1; ○, MR-481.

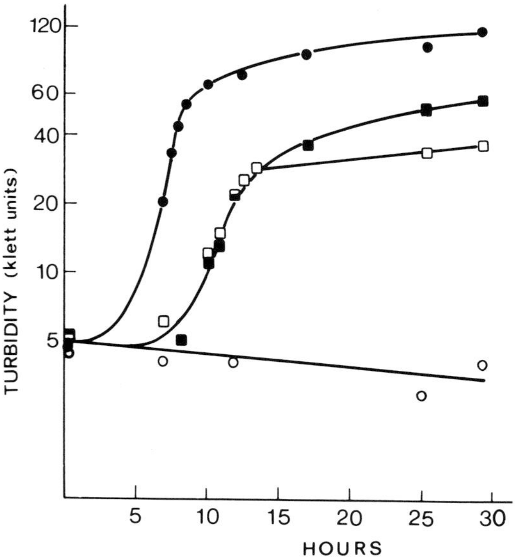

FIG. 4. Growth of RAG-1 and derivative strains in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer were inoculated with RV-1 (□), RV23-4 (■), RAG-1 (●), or MR-481 (○). The tubes were overlaid with 0.5 ml of hexadecane and incubated with shaking at 30°C.

1:50 in fresh hexadecane growth medium. After incubation for 48 h, the culture was streaked onto nutrient agar plates; among eight colonies which were isolated and tested for adherence to hexadecane, one clone, termed RV23-4, showed significantly increased adherence and was studied further in this investigation.

Adherence properties of partial revertants RV-1 and RV23-4. The adherence to hydrocarbon of strains RAG-1, MR-481, RV-1, and RV23-4 is shown in Fig. 1. Strain RV-1 adhered only slightly better to hexadecane and xylene than the nonadherent mutant MR-481, whereas RV23-4 adhered considerably better to all three hydrocarbons tested. Both partial revertant strains adhered to polystyrene (Fig. 2).

Growth of strains RV-1 and RV23-4 on hexadecane. Growth of RAG-1, MR-481, and partial revertants RV-1 and RV23-4 was compared on acetate and hexadecane media. In the presence of the soluble carbon and energy source (sodium acetate), all four strains grew with similar kinetics (Fig. 3). However, when hexadecane served as the sole carbon and energy source, growth of the four strains corresponded to their adherence ability (Fig. 4). RAG-1 grew, after a brief lag, with an apparent doubling time of ca. 1 h, reaching 115 Klett units after 34 h of incubation. Nonadherent mutant MR-481 cells were unable to grow under these conditions. Partial adherence revertants RV-1 and RV23-4 grew with similar kinetics: after a lag of about 7 h, growth commenced, with an apparent doubling time of ca. 2 h. Strain RV23-4 grew to a higher turbidity than RV-1, reaching 57 Klett units after 34 h, as opposed to 37 Klett units obtained for RV-1 cells. It should be emphasized that inocula of all four strains were prepared in the same manner as for measurement of adherence (Fig. 1).

Agglutinability. Large differences were found in the ability of RAG-1 and the derivative strains

TABLE 1. Agglutination kinetics of RAG-1 and derivative strains by antibodies raised against RAG-92 cells[a]

Strain	% Agglutination[b] after:		
	6 h	22 h	28 h
RAG-1	9	69	84
MR-481	3	7	8
RV-1	89	96	95
RV23-4	80	98	92
RAG-92	9	53	67
AB15	0	1	3

[a] Antibody was obtained from rabbits inoculated with intact RAG-92 cells. Cells were grown to stationary phase on brain heart infusion broth, washed, and suspended in PUM buffer to an initial absorbance at 400 nm of 0.9 to 1.1.

[b] Percent decrease in optical absorbance at 400 nm.

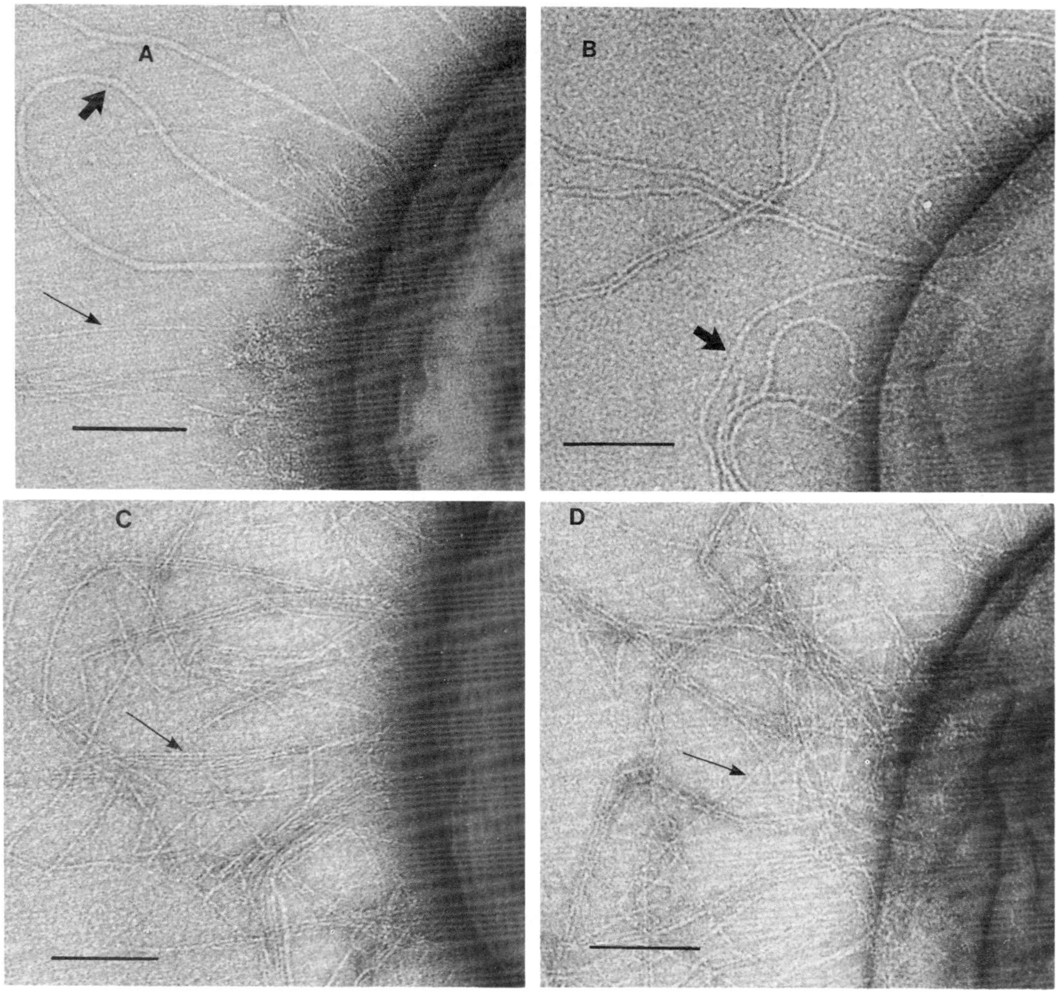

FIG. 5. Electron micrographs of uranyl acetate-stained RAG-1, MR-481, RV-1, and RV23-4. Cells were grown to stationary phase in brain heart infusion broth. (A) RAG-1; (B) MR-481; (C) RV-1; (D) RV23-4. Bars, 0.1 µm. Thin arrows designate thin fimbriae; thick arrows designate thick fimbriae.

to agglutinate in the presence of antibodies raised against intact cells (Table 1). RAG-1 and its lysine auxotroph, RAG-92, agglutinated with similar kinetics. Nonadherent mutant MR-481 resembled nonagglutinating mutant AB15 in its inability to agglutinate in the presence of antibody. In contrast, both partial adherence revertants, RV-1 and RV23-4, agglutinated much more rapidly than wild-type RAG-1 cells.

Presence of thin fimbriae. RAG-1 and derivative strains were examined for the presence of fimbriae by electron microscopy after growth on complex medium. Wild-type RAG-1 cells were observed to possess two major fimbrial types: thin fimbriae, ca. 3.5 nm in diameter, and thicker fimbriae, ca. 6.5 nm in diameter (Fig. 5a). In contrast to RAG-1, nonadherent mutant MR-481 was almost completely devoid of the thin fimbriae (Fig. 5B). Among over 100 MR-481 cells which were closely scrutinized in seven separate experiments, thin fimbriae were absent on most of the cells. On a few cells, up to five thin fimbriae resembling those present on RAG-1 were observed, as opposed to several hundred present on wild-type cells. Thick fimbriae were present on MR-481 cells. Both partial revertant strains, RV-1 and RV23-4, were covered with a dense layer of thin fimbriae resembling those present on RAG-1 (Fig. 5C and D). A comparison of numerous electron micrographs demonstrated that both partial revertants possessed a much larger number of thin fimbriae than did wild-type RAG-1 cells. Very few fimbriae of the thick variety were observed on the two partial revertant strains. The distribution of thin fimbriae on the surfaces of the four strains after

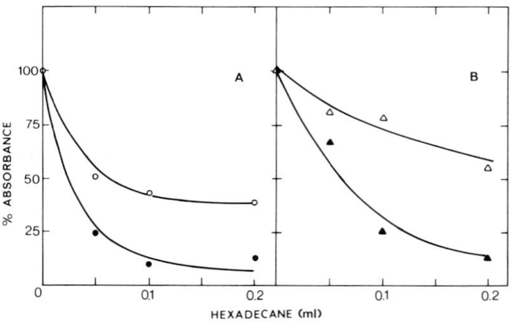

FIG. 6. Effect of shearing on adherence of RAG-1 (A) and RAG-92 (B) to hexadecane. Cells were harvested after 48 h of growth on ethanol medium, washed twice, and suspended in PUM buffer to ca. 1,000 Klett units. Cells, sheared for 5 min in a Sorvall Omnimixer, were harvested and assayed for adherence to hexadecane. Symbols: ○, RAG-1 cells subjected to shearing; ●, nonsheared RAG-1 cells; △, RAG-92 cells subjected to shearing; ▲, nonsheared RAG-92 cells.

growth on minimal ethanol medium was similar to that described above; however, thick fimbriae were absent in all cases.

Effect of shearing treatment on adherence to hexadecane. Adherence of RAG-1 and RAG-92 cells to hexadecane was significantly reduced by exposing cells to shearing in a Sorvall Omnimixer (Fig. 6). With 0.2 ml of hexadecane, the adherence of sheared RAG-1 and RAG-92 cells was 62 and 45%, respectively, whereas the untreated cells of both RAG-1 and RAG-92 adhered to the extent of 87%. No damage to the cells after this treatment was observable by phase microscopy.

CIE of supernatant fluids obtained from the shearing procedure. Various cell surface antigens of RAG-1 have been identified with CIE (E. A. Bayer, E. Skutelsky, S. Goldman, E. Rosenberg, and D. L. Gutnick, submitted for publication). Since the supernatant fluid obtained after shearing of RAG-1 cells presumably contained adherence factor(s) released from the cell surface by this treatment, derivative strains MR-481, RV-1, and RV23-4 were similarly subjected to shearing, and the supernatant fluids were run against antibody raised against RAG-92 cells (Fig. 7). The supernatant fluids of strains RAG-1, RV-1, and RV23-4 gave a broad precipitation band, corresponding to band C3, identified as the surface component absent from mutant AB15 cells (E. A. Bayer, E. Skutelsky, S. Goldman, E. Rosenberg, and D. L. Gutnick, submitted for publication). This band was not observed in the corresponding supernatant fluid of sheared MR-481 cells.

Adherence properties of nonagglutinating strain AB15. In addition to lacking cell surface component C3, nonagglutinating mutant AB15 is also completely devoid of thin fimbriae after growth on both complex and minimal ethanol media. On the basis of these observations, strain AB15 was examined for adherence to hexadecane. After growth on brain heart infusion broth, AB15 adhered similarly to hexadecane as compared with wild-type RAG-1 cells (data not shown). Furthermore, AB15 cells grown on nutrient agar similarly adhered to polystyrene (Fig. 2). However, AB15 cells harvested from minimal ethanol medium resembled MR-481 in their inability to adhere to either polystyrene or liquid hydrocarbon, as compared with the parental strains (Fig. 8).

Growth of AB15 on hexadecane. Growth of AB15 and its parent strain, RAG-92, on acetate and on hexadecane media was carried out as described in the legends to Fig. 3 and Fig. 4, respectively, except that lysine (50 μg/ml) was present in the growth media. When sodium acetate served as the carbon and energy source, both strains grew with similar kinetics (Fig. 9A). In hexadecane medium, RAG-92 grew after a lag of about 20 h. In contrast, AB15 was completely unable to grow for at least 140 h (Fig. 9B). Strain AB15 failed to grow on hexadecane in liquid medium under the conditions described, regardless of whether the inoculum consisted of adherent (complex medium-grown) or nonadherent (minimal ethanol medium-grown) AB15 cells. Both AB15 and RAG-92 grew on minimal agar plates supplemented with lysine (50 μg/ml) and hexadecane vapors.

DISCUSSION

In a previous report, the inability of nonadherent mutant MR-481 to grow on hexadecane under conditions of low initial cell density and limited emulsification was demonstrated. The interpretation of these data was that adherence is crucial for growth of RAG-1 on hexadecane under these conditions (14).

The present report extends these findings and focuses on the role of thin fimbriae in mediating adherent properties of RAG-1 and in growth on hexadecane. The data implicating the thin fimbriae in adherence to hydrocarbons are the following: (i) nonadherent mutant MR-481 lacks the thin fimbriae; (ii) mutants RV-1, RV-2, RV-3, RV-4, and RV23-4, which regained the ability to adhere to polystyrene and partially regained the ability to adhere to hydrocarbons, all exhibited reappearance of the thin fimbriae; (iii) AB15, a mutant which was independently isolated for its inability to agglutinate in the presence of antibody (1) and lacks thin fimbriae, was nonadherent after growth in minimal ethanol medium; (iv) shearing treatment, a common

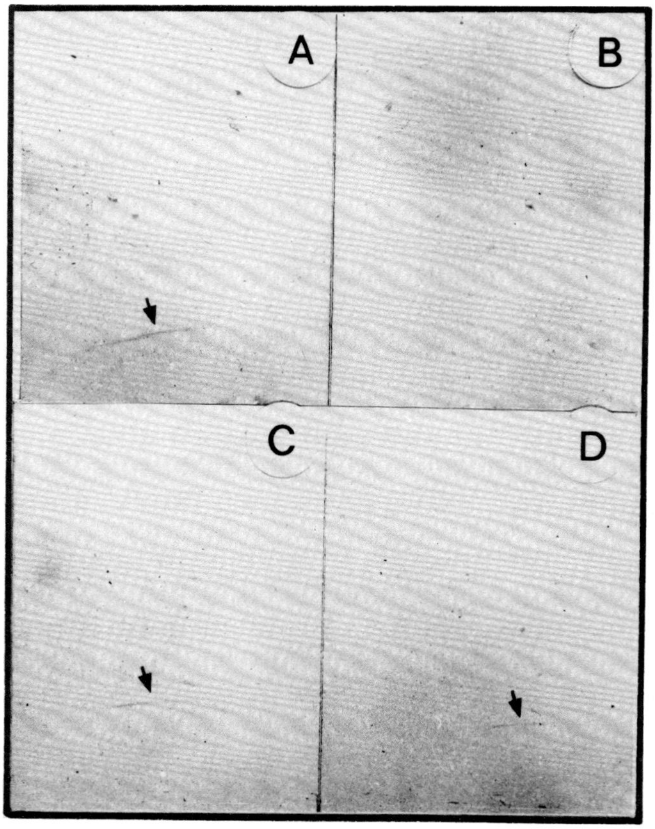

FIG. 7. CIE of sheared supernatant fluids. Concentrated samples of supernatant fluid of cells sheared after growth on ethanol medium were prepared as described in the text. Samples: (A) RAG-1; (B) MR-481; (C) RV-1; (D) RV23-4. The presence of bands is indicated by arrows. In all cases, the anode is to the right in the first dimension and upward in the second dimension.

method of removing fimbriae (3), resulted in a marked decrease in the ability of RAG-1 and RAG-92 cells to adhere to hexadecane; the only RAG-1 antigen released by this procedure which could be observed by CIE corresponded to band C3, previously observed by Bayer et al. (E. A. Bayer, E. Skutelsky, S. Goldman, E. Rosenberg, and D. L. Gutnick, submitted for publication); (v) band C3 was present in samples obtained from fimbriated wild-type and revertant strains but was absent in both thin fimbriae-less strains, MR-481 and AB15. It should be noted that none of the data presented here eliminates the possibility that the fimbriae are carriers of components which mediate these adherence phenomena.

Bacterial fimbriae have been implicated in a wide variety of adherence phenomena, including pellicle formation (7), attachment to sulfur (18), and adherence to mammalian, plant, and fungal cells (7). The presence of fimbriae has been linked to pathogenesis in various strains, attesting to the importance of bacterial adherence for colonization of host tissues (7).

Various hydrophobic properties have been associated with bacterial fimbriae. Fimbriae contribute to the ability of various bacterial cells to be retained on hydrophobic gels (4, 16); certain fimbriae are able to adhere to hydrophobic surfaces such as polystyrene beads (2). Proteins comprising various fimbriae have been shown to contain a high proportion of hydrophobic amino acid residues; moreover, hydrophobic amino acid sequences have been reported for *Neisseria, Moraxella*, and *Pseudomonas* fimbriae (7). To our knowledge, this is the first evidence demonstrating participation of bacterial fimbriae in adherence to hydrocarbons.

The inability of AB15 to adhere to hexadecane after growth on ethanol suggests that the thin fimbriae constitute the main adherence factor of RAG-1 cells grown on ethanol medium. However, thin fimbriae are not the sole surface component which mediates the adherence of RAG-1 strains to hydrocarbons and polystyrene. AB15 cells grown in complex medium adhered to hexadecane and polystyrene despite the absence of thin fimbriae on these cells.

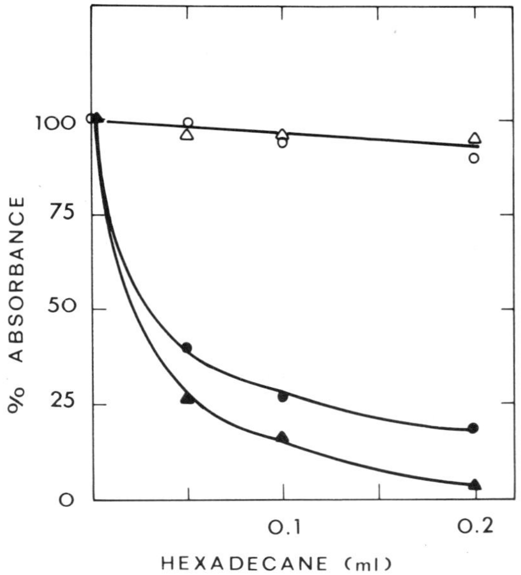

FIG. 8. Adherence of AB15 and MR-481 to hexadecane after growth on ethanol minimal medium. Strains AB15 (○) and MR-481 (△) and their respective parental strains, RAG-92 (●) and RAG-1 (▲), were compared for adherence to hexadecane.

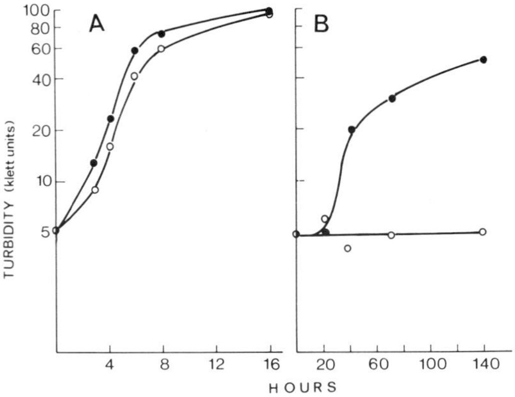

FIG. 9. (A) Growth of AB15 (○) and RAG-92 (●) in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth and inoculated into 4.2 ml of acetate medium supplemented with 50 μg of lysine per ml in Klett tubes. (B) Growth of AB15 and RAG-92 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer supplemented with 50 μg of lysine per ml were inoculated with either RAG-92 (●) or AB15 (○). The tubes were overlaid with 0.5 ml of hexadecane and incubated as described in the text.

A comparison of the growth of AB15 and RAG-92 on hexadecane medium demonstrated the inability of AB15 to grow on hexadecane medium under conditions of low initial cell density and limited emulsification of hydrocarbon. The correlation between growth on hexadecane and presence of thin fimbriae is even more striking if one considers that AB15 was not selected as a mutant deficient in adherence or growth on hydrocarbons, but rather on the basis of its inability to interact with specific antibodies (1). As in the case of MR-481 (14), the inability to AB15 to grow on hexadecane medium was not due to a faulty hydrocarbon-metabolizing system; AB15 grew normally on minimal plates supplemented with lysine and hexadecane vapors. The inability of AB15 to grow on hexadecane medium, even when the inoculum consisted of adherent cells, suggests that (i) adherence per se is a necessary but insufficient requirement for growth of RAG-1 in hexadecane medium and (ii) the thin fimbriae constitute the crucial adherence factor allowing growth of RAG-1 on hexadecane under these conditions. Studies are under way to further test the relationship between thin fimbriae and growth of RAG-1 on hydrocarbon.

The fact that strain RV-1 adhered relatively poorly to liquid hydrocarbons, even though it possessed an abundance of thin fimbriae, may be explained by one of the following possibilities: (i) although the thin fimbriae appear similar to those of the wild-type RAG-1 cells, they are altered in a manner which does not radically affect their shape or immunological properties, but reduces adherence to hydrocarbon; (ii) the large number of thin fimbriae present on RV-1 cells, as opposed to the wild type, interferes with adherence to hexadecane, perhaps due to increased interactions between the fimbriae themselves (A. Perry and I. Ofek, unpublished data).

The strong correlation which was established between the distribution of thin fimbriae among the various strains and their relative agglutinability demonstrates that the thin fimbriae are the major agglutinogen of RAG-1 cells. The results obtained with CIE extend the previous results of Bayer et al. (1) and further demonstrate the involvement of thin fimbriae in antibody-mediated agglutination of RAG-1.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Bayer, E. A., E. Rosenberg, and D. Gutnick.** 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. J. Gen. Microbiol. **127:**295–300.
2. **Brinton, C. C.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. Ser. II **27:**1003–1054.
3. **Eshdat, Y., F. J. Silverblatt, and N. Sharon.** 1981. Disso-

ciation and reassembly of *Escherichia coli* type 1 pili. J. Bacteriol. **148**:308–314.

4. **Faris, A., T. Wadström, and J. H. Freer.** 1981. Hydrophobic adsorptive and hemagglutinating properties of *Escherichia coli* possessing colonization factor antigens (CFA/I or CFA/II), type 1 pili, or other pili. Curr. Microbiol. **5**:67–72.
5. **Henrichsen, J., and J. Blom.** 1975. Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. Acta Pathol. Microbiol. Scand. Sect. B **83**:103–115.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Pearce, W. A., and T. M. Buchanan.** 1980. Structure and cell membrane-binding properties of bacterial fimbriae, p. 291–344. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, London.
8. **Reisfeld, A., E. Rosenberg, and D. Gutnick.** 1972. Microbial degradation of crude oils: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. **24**:363–368.
9. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
10. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42**:375–377.
11. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.
12. **Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg, and I. Ofek.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. **33**:29–33.
13. **Rosenberg, M., E. Rosenberg, and D. Gutnick.** 1980. Bacterial adherence to hydrocarbons, p. 541–542. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.
14. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane. J. Bacteriol. **148**:51–57.
15. **Savage, D. C.** 1980. Adherence of normal flora to mucosal surfaces, p. 31–60. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, London.
16. **Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22**:462–472.
17. **Weeke, B.** 1973. Crossed immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):47–56.
18. **Weiss, R. L.** 1973. Attachment of bacteria to sulphur in extreme environments. J. Gen. Microbiol. **77**:501–507.

Chapter 17

MEL ROSENBERG
School of Dental [illegible]
Tel Aviv [illegible]
Ramat-Aviv, [illegible]

Microbial Enhanced Oil Recovery

J.E. Zajic
D.G. Cooper
T.R. Jack
N. Kosaric

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Tulsa, Oklahoma

1983

17

BACTERIAL ADHERENCE TO HYDROCARBONS

M. Rosenberg, D.L. Gutnick, and E. Rosenberg
Tel Aviv University

The importance of cell adherence in growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces.[1] The initial step in the life cycle of *Bdellovibrio* is attachment to Gram-negative bacteria.[2,3] Specific cell-to-cell interactions pervade the entire life cycle of the myxobacteria.[4,5] Adhesion is a prerequisite to the successful colonization by microorganisms of animals and plants.[6,7] Microbial adhesion to surfaces has been the subject of several recent reviews.[6,7,8,9,10]

In cases where the carbon or energy source is a water-insoluble material, such as cellulose or chitin, bacterial adhesion can facilitate growth.[10,11,12] But cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, growth of microorganisms on hydrocarbons presents a special problem since hydrocarbons are immiscible with water and their breakdown cannot occur extracellularly. The first step in aromatic or aliphatic hydrocarbon degradation is introduction of molecular oxygen into the molecules by cell-associated enzymes.[13,14,15]

Two general types of hydrocarbon-cell interactions have been postulated, depending upon the state and size of the oil droplets relative to the size of the microbial cells: (*i*) adherence of cells to large oil droplets and (*ii*) pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets.[16] The relative contribution of these two types of interactions to the growth of bacteria on hydrocarbons has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon-aqueous interfacial area and growth rate can be used to support either hypothesis.[17]

Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which bacterial hydrophobicity and adherence to hydrophobic surfaces are involved. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in partitioning microorganisms at the air-water interface;[18,19] in the adherence of bacteria to nonwettable plastic surfaces,[20,21,22,23] and in the attachment of bacteria to phagocytes and other mammalian cells.[24,25,26,27,28]

A rapid and quantitative method for measuring bacterial adherence to hydrocarbons was described previously.[29,30] The present report deals with the use of this technique in (*i*) examining the cell surface hydrophobicity of various bacterial species, some of which do not metabolize hydrocarbons, and (*ii*) selecting and isolating nonadherent mutants. The isolation of a nonadherent mutant of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 has enabled us to examine the role of adherence in bacterial growth on hexadecane.

MATERIALS AND METHODS

Bacterial Strains. Tables 1 and 2 contain a list of bacteria used in this study. *Acinetobacter calcoaceticus* MR-481 is a nonadhering derivative of strain RAG-1.

Media and Growth Conditions. For measuring adherence to test hydrocarbons or preparing inocula for growth experiments, 0.1 ml of bacteria from an overnight culture was inoculated into 50 ml of growth medium and was incubated in a New Brunswick model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested at the end of the

Table 1. Adherence of bacteria to hexadecane.

Strain	Source	Growth Medium[a]	Adherence to Hexadecane (%)[b]
Acinetobacter calcoaceticus RAG-1	this laboratory	BHI	97
Bacillus cereus	local isolate	NB	1
Bacillus subtilis 168	E.Z. Ron	NB	0
Enterobacter aerogenes CDC 659/66	E.Z. Ron	NB	9
Escherichia coli B	E.Z. Ron	NB	0
Escherichia coli K12	E.Z. Ron	NB	2
Escherichia coli J5 (rough)	E.Z. Ron	NB	11
Micrococcus lysodeikticus	I. Friedberg (ATCC 4698)	NB	0
Myxococcus xanthus	this laboratory	CT	35
Proteus mirabilis S1959	S. Rottem	NB	0
Pseudomonas aeruginosa PAS279	J. Shapiro	NB	0
Serratia marcescens	R. Zack	NB	96
Staphlococcus albus	R. Zack	NB	0
Staphlococcus aureus	R. Zack	NB	91
Streptococcus pyogenes M5	I. Ofek	BHI	84

[a] Cells were harvested at the end of the exponential phase

[b] The standard adherence assay was performed with 0.2 ml hexadecane.

exponential phase, washed twice, and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_3O$; 7.26 g KH_2PO_4; 0.8 g urea; 0.2 g $MgSO_4 \cdot 7H_2O$; and distilled water to 1,000 ml, pH 7.1). Growth media were BHI, brain heart infusion broth (Difco); hexadecane medium, PUM buffer supplemented with 0.2% (v/v) hexadecane (olefin free, 99% purity, Fluka, Switzerland); P-hexadecane medium, 5 g NH_4NO_3, 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 ml hexadecane, 50 mg threonine, and tap water to 1,000 ml; and NB, nutrient broth (Difco) supplemented with 0.5% NaCl. CT medium contained 1% casitone (Difco) and 0.2% $MgSO_4 \cdot 7H_2O$[31].

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14-mm diameter) containing 4.2 ml of an aqueous medium to give an initial turbidity of 5 Klett units. The acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml hexadecane (olefin free, 99% purity, Fluka, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick gyrotory water bath shaker (Model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Adherence to Hydrocarbons. The technique for measuring adherence of bacteria to hydrocarbons has been

Table 2. Adherence of *Acinetobacter* strains to hexadecane.

Strain Designation	Source	Growth on Hexadecane[a]	Adherence to Hexadecane (%)[b]
RAG-1 (ATCC 13012)	this laboratory	+	82
HO1-N	W.R. Finnerty	+	99
BD4	E. Juni	+	0
BD413	E. Juni	+	4
B5W str 71 (ATCC 15149)	K. Bryn	+	84
CDC 7827 (ATCC 17959)	K. Bryn	+	86
B5W (ATCC 17903)	K. Bryn	−	0
ATCC 17906	K. Bryn	−	52
ATCC 17977	K. Bryn	−	7
ATCC 17985	K. Bryn	−	41

[a] Growth was examined on PUM agar in the presence of hexadecane vapors.

[b] Adherence was measured on cells grown to stationary phase on BHI broth; the standard adherence assay was performed with 0.2 ml hexadecane.

described previously.[32] Cells were washed twice and were resuspended in PUM buffer to an initial A_{400} of approximately 1.5. Various volumes of the test hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom, acid-washed test tubes (10-mm diameter) containing 1.2 ml of washed-cell suspension, and the phases were vortexed uniformly for 120 sec. After allowing 15 min for the phases to separate, the absorbance of the lower aqueous phase of each tube was measured at 400 nm (Gilford model 240 spectrophotometer, 1-cm light path). Adherence was calculated as the percentage loss in absorbance relative to that of the initial cell suspension.

Isolation of the Nonadherent Mutant MR-481. A spontaneous mutant of RAG-1 that is deficient in its ability to adhere to hydrocarbons was isolated by the following technique: RAG-1 cells were inoculated into 50 ml of a nutrient broth medium (NB) and were incubated in a New Brunswick Model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested in stationary phase following 18 hr growth, washed twice, and resuspended in PUM buffer to an A_{400} of approximately 1.5. Two ml of n-octane were added to 5 ml of the cell suspension, and the phases were vortexed for 5 min. Following phase separation the lower aqueous phase was tranferred to a second test tube. Octane was again added, and the phases were mixed as described previously. This extraction process was continued until cells in the lower aqueous phase were too few to be visible under microscopic examination. The final aqueous phase served as the inoculum for a second nutrient broth culture that was grown to stationary phase as previously described. Cells were again harvested, washed, and extracted with octane, and the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. The final aqueous phase was plated on nutrient agar. Three individual colony types were isolated and tested for adherence to hydrocarbons. One clone of cells, termed MR-481, exhibited no significant affinity towards the test hydrocarbons and was chosen for further study.

Emulsan Preparation. The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously.[33] The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH Chem. Ltd.), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing extensively against distilled water. The material was then deproteinized by the phenol method and dialyzed.[34] The final product had a reduced viscosity of 490 cc per g, an emulsifying activity of 150 units per mg, an ester content of 0.5 μ moles per mg, and a protein content of less than 0.5%. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and was sterilized by autoclave prior to its addition in the growth experiments.

RESULTS

Bacterial Adherence to Hydrocarbons. The basic assay for bacterial adherence to hydrocarbons is illustrated in

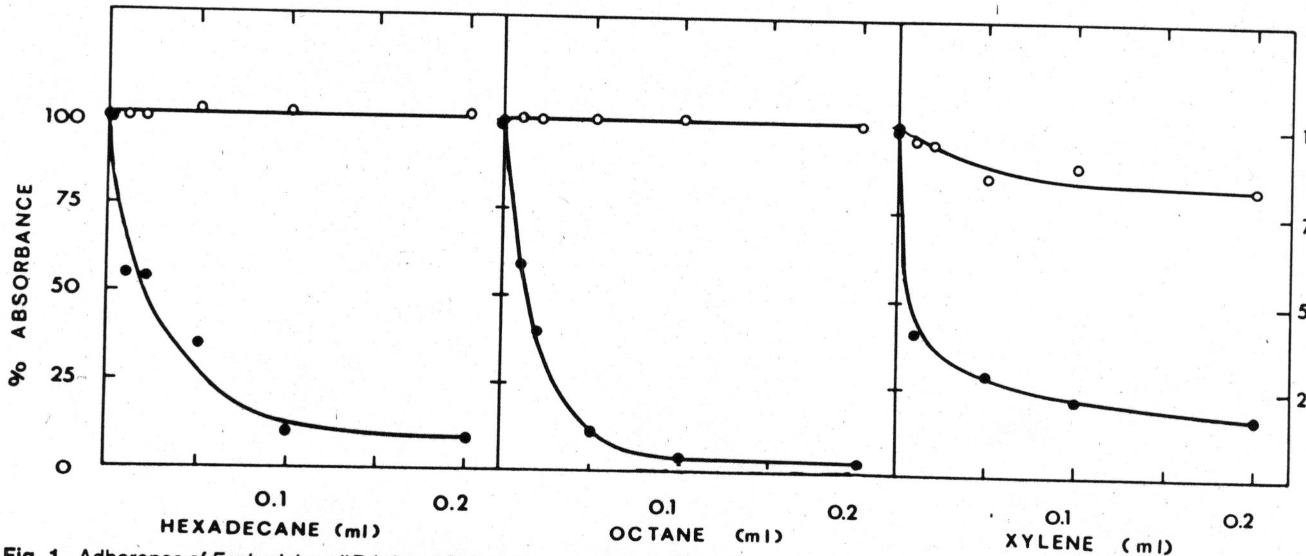

Fig. 1. Adherence of *Eschericia coli* B (○) and *Acinetobacter calcoaceticus* RAG-1 (●) to hydrocarbons. Cells were harvested at the end of the exponential growth phase, washed twice in PUM buffer, and then resuspended to an initial A_{400} of 1.2–1.4. The adherence assay was performed as described in "Materials and Methods." The ordinate is the percentage of initial absorbance of the cell suspension

Figure 1. The assay was performed with varying volumes of three test hydrocarbons: n-hexadecane, n-octane, and p-xylene. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbons (e.g., *E. coli*), the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested (e.g., *A. calcoaceticus* RAG-1), a creamy upper layer was obtained following mixing. Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was a function of volume of hydrocarbon added. The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5% isopropanol resulted in immediate droplet coalescence and the concommitant release of extracted cells into the aqueous phase, thereby demonstrating that the bacteria had partitioned to the hydrocarbon-water interface.

Tables 1 and 2 summarize a number of experiments in which cell surface hydrophobicity of a variety of bacteria was measured by adherence to hexadecane. The majority of laboratory strains examined exhibited low affinity toward the test hydrocarbon. Among the bacterial strains exhibiting high affinity for hexadecane were hydrocarbon-degrading *Acinetobacter calcoaceticus* strains RAG-1, HO1-N, B5W str 71, and CDC 7828; and bacteria that do not metabolize hydrocarbons: *Serratia marcescens, Staphylococcus aureus*, and *Streptococcus pyogenes*.

Pseudomonas aeruginosa (which can utilize hexadecane as the sole carbon and energy source) exhibited no significant affinity toward any of the test hydrocarbons when grown to late exponential phase in nutrient broth (Figure 2). Since adherence in this organism might have required the presence of hydrocarbons during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane (Figure 2). The affinity of alkane-grown *P. aeruginosa* toward hexadecane increased somewhat, e.g., 32% of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane. In contrast, *A. calcoaceticus* RAG-1 exhibited much higher affinity when grown on hexadecane; 93% of the cells could be removed by as little as 0.05 ml hexadecane.

Role of Adherence in the Growth of A. calcoaceticus *RAG-1 on Hexadecane. Acinetobacter calcoaceticus* RAG-1 adheres avidly to hydrocarbons.[35,36,37] A spontaneous mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells that did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Figure 3).

Mutant MR-481 resembled the wild type in the following characteristics: (*i*) colonial and cell morphology,

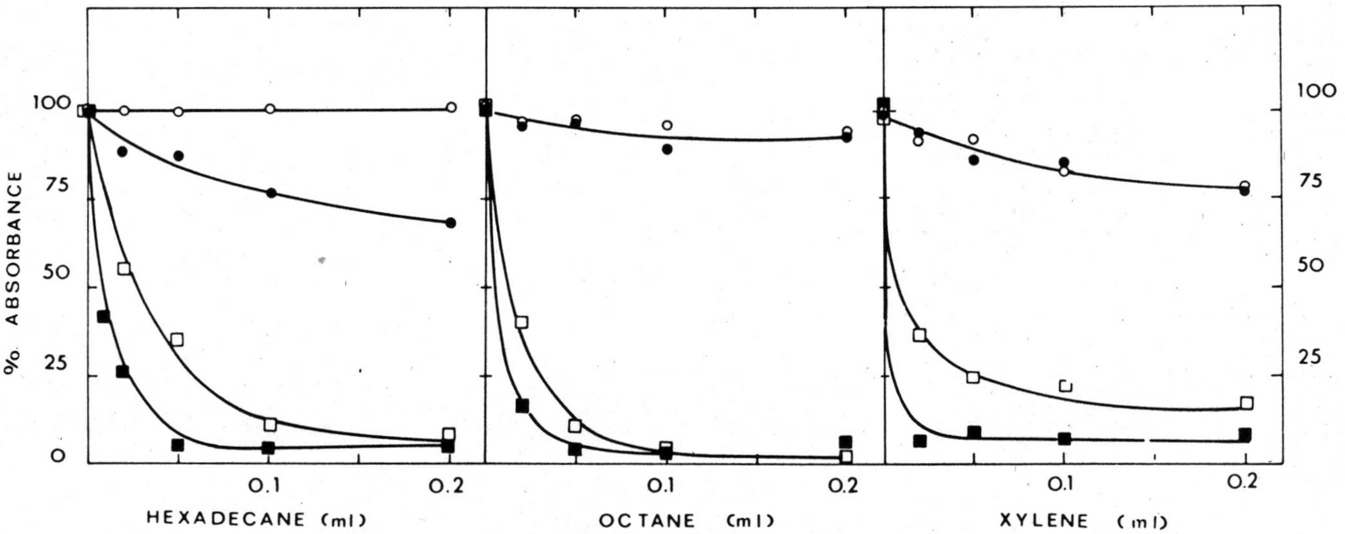

Fig. 2. Adherence of *Pseudomonas aeruginosa* PAS 279 and *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons. The experiment was performed as described in Figure 1 with cells harvested at the end of the exponential growth phase: *P. aeruginosa*, NB medium (○), P-hexadecane medium (●); *A. calcoaceticus*, NB medium (□), hexadecane medium (■)

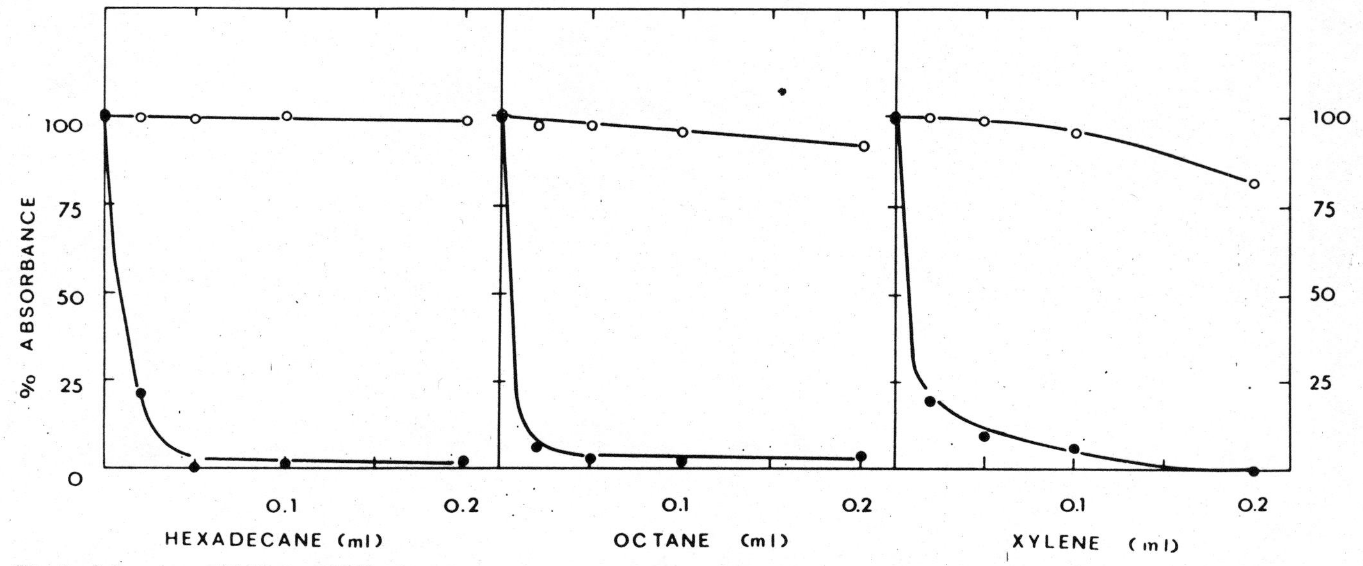

Fig. 3. Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth, washed twice, and resuspended in PUM buffer to an A_{400} = 1.45 to 1.50. To 1.2 ml of suspended RAG-1 (●) or MR-481 (○) cells were added various volumes of test hydrocarbon, and the mixtures were vortexed for 120 sec. Following phase separation the turbidity of the lower aqueous phase was measured. The ordinate is the percent of initial absorbance of the cell suspension

(*ii*) sensitivity to two bacteriophages, ap2 and ap3, that are specific for *A. calcoaceticus* RAG-1 strains,[38] (*iii*) production of extracellular emulsifying activity during growth on ethanol as the sole carbon and energy source,[39] and (*iv*) doubling time on brain heart infusion broth (38 min at 30°C with aeration).

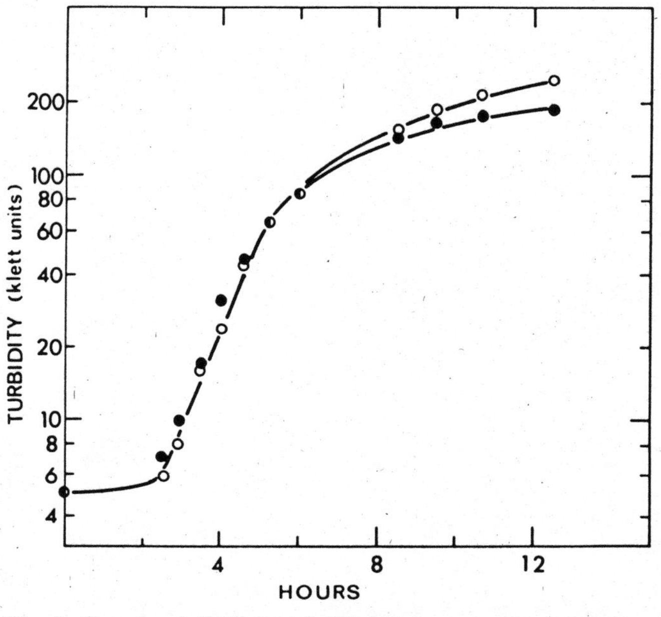

Fig. 4. Growth of RAG-1 and MR-481 in an acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice, and resuspended in PUM buffer supplemented with 0.2% sodium acetate. RAG-1 (●) and MR-481 (○) cells were inoculated into a 4.2-ml acetate medium in Klett tubes to an initial turbidity of 5 Klett units

In all growth experiments, inocula were prepared in the same manner as was described for measuring adherence in Figure 3. In a defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Figure 4). Following a 2-hr lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew, whereas the nonadherent mutant MR-481 failed to grow for at least 54 hr (Figure 5). Growth of RAG-1 was accompanied by breaking the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells. Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus, the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since attached bacteria were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Figure 6).

Effect of Emulsan on the Growth of MR-481 on Hexadecane. *A. calcoaceticus* RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan.[40,41,42,43] It was of interest to examine the effect of a highly purified preparation of emulsan (see "Materials and

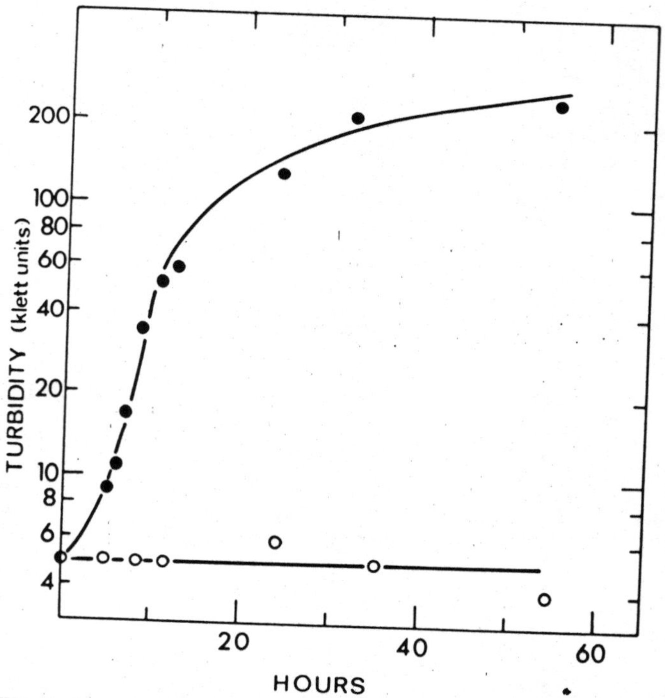

Fig. 5. Growth of RAG-1 and MR-481 in a hexadecane medium. Klett tubes containing 4.2 ml PUM buffer were inoculated with either RAG-1 (●) or MR-481 (○) to an initial turbidity of 5 Klett units. Tubes were overlayed with 0.5 ml hexadecane and were incubated as described in "Materials and Methods"

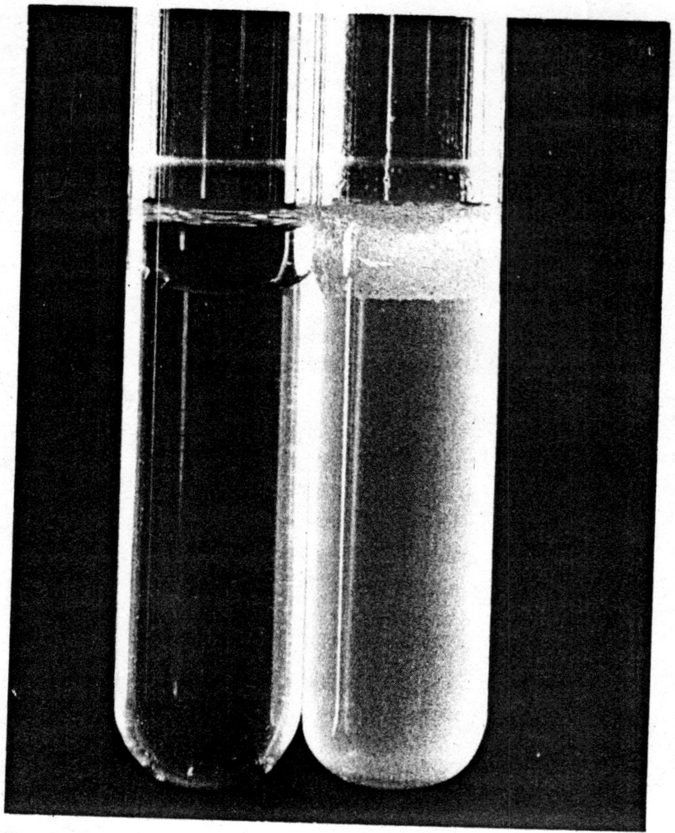

Fig. 6. Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. Tubes were photographed following 48 hr of incubation under the conditions described in Figure 3

Methods") on the growth on hexadecane of RAG-1 and the nonadherent mutant MR-481 (Figure 7). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481. However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both emulsan and hexadecane began after about 6 hr, whereas neither component alone supported growth for 54 hr. At the weight ratio of hexadecane-emulsan used (1,925:1), the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Figure 8). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to adding the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic nor phenotypic reversion of MR-481 from nonadherent to adherent cells. As shown in Table 3, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan compared to the initial inoculum grown in a rich medium (Figure 3).

RAG-1 cells that were harvested and washed following growth on hexadecane both in the presence and absence of emulsan exhibited high affinity toward hexadecane (Table 3). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

DISCUSSION

Mudd and Mudd reported over fifty years ago that certain microorganisms tend to concentrate at oil-water interfaces.[44] Although this observation has been corroborated frequently during studies on the growth of microorganisms on liquid hydrocarbons, there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons.[45,46,47,48,49,50] Certain investigators have argued

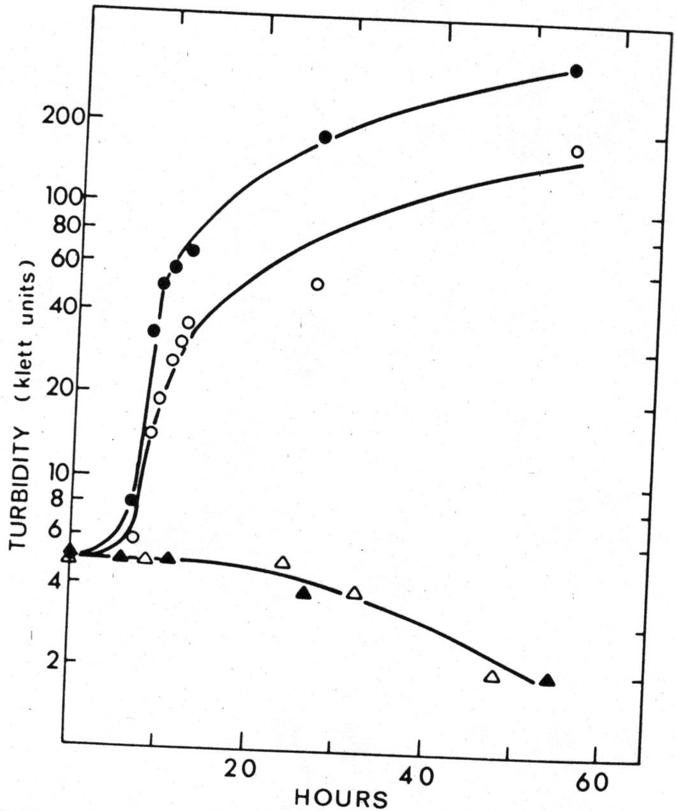

Fig. 7. Effect of emulsan on the growth of MR-481 on hexadecane. Inocula preparation and incubation conditions were as described in Figure 3. RAG-1 (●) and MR-481 (○) were grown on a hexadecane medium in the presence of 50 μg/ml emulsan. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg/ml emulsan in the absence of hexadecane

Table 3. Adherence of RAG-1 and MR-481 following growth on hexadecane.

Strain	Turbidity (A_{400})[a]		% Adherence
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR-481[c]	1.43	1.36	4.9

[a] Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture was vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested following 54 hr growth on a hexadecane medium, washed twice, and resuspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested following 54 hr growth on a hexadecane medium in the presence of 50 μg/ml emulsan, washed twice, and resuspended in PUM buffer.

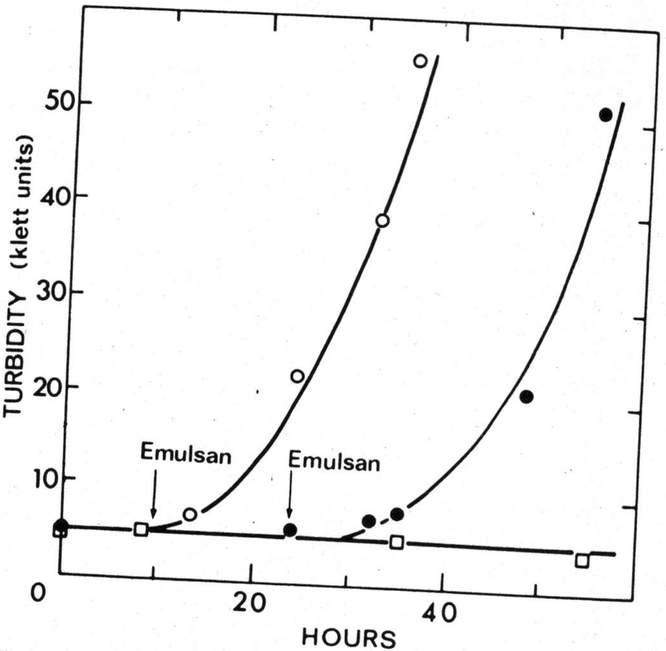

Fig. 8. Induction of growth of MR-481 on hexadecane by emulsan addition. Inocula preparation and incubation conditions were as described in Figure 3. Emulsan was added to a final concentration of 50 μg/ml at 10 hr (○) or 24 hr (●): Tubes containing no emulsan served as controls (□)

that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets.[51,52,53] *A. calcoaceticus* RAG-1 is an interesting microorganism in this regard because it adheres avidly to hydrocarbons and produces an extracellular emulsifying agent, emulsan, that has been extensively characterized.[54,55,56,57,58,59,60]

Isolation of a mutant strain of *A. calcoaceticus* RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The nonadherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions that supported growth of wild-type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane — insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system — were excluded by the following data: (*i*) MR-481 and RAG-1 cells grew with similar kinetics on an acetate medium under conditions identical to those employed for growth on hexadecane; (*ii*) MR-481 was able to grow on a hexadecane medium following addition of an emulsifier; and (*iii*) MR-481 cells were able to grow on a solid medium supplemented with hexadecane vapors as the sole carbon and energy source. We conclude from these data that adherence is a prerequisite for growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce a potent extracellular emulsifying agent that enables growth of nonadhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon.[61,62,63] At the low inoculum employed (5×10^7 cells/ml), the concentration of emulsan that the cells produced was too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of nonadherent bacteria on hydrocarbons. The poor adherence of *P. aeruginosa* PAS 279 to hydrocarbon (Figure 2) suggests that its growth, similar to that of MR-481, depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments conditions of low cell density and limited emulsification prevail; thus, the growth of poorly adhering Pseudomonas strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation probably does not reflect growth in the open environment, where emulsifiers and dispersed hydrocarbons would tend to diffuse away from the cell.[64,65,66,67]

The adherence to hydrocarbon of bacteria that do not metabolize hydrocarbons (Tables 1 and 2) can be explained in terms of general cell surface hydrophobicity.[68,69] *S. pyogenes*, for example, has been reported to adhere to buccal epithelial cells through hydrophobic interactions.[70] Its adherence to hydrocarbons may thus be mediated by such interactions. Similarly, hydrophobic cell surface components that enable *Serratia* to partition at the air-water interface may also promote their adherence to hydrocarbons as observed in this study.[71]

Bacterial hydrophobicity has been cited as an important factor in several biological phenomena, including bacterial pathogenicity,[72] and in adherence and growth of bacteria on various hydrophobic surfaces.[73,74,75,76] Adherence to hydrocarbons provides a simple quantitative technique for measuring cell surface hydrophobicity and can be used for the selection and isolation of nonadhering mutants. Adherence of RAG-1 to hydrocarbons may reflect a general phenomenon in microbiology: a biological advantage is conferred on microbial cells that can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface that provides nutrition and enables growth.

SUMMARY

A simple, quantitative assay method was used to measure the adherence of a variety of bacteria to liquid hydrocarbons. Bacterial species differed greatly in their ability to adhere to hydrocarbons; even within the same species, *Acinetobacter calcoaceticus*, different strains varied greatly in their cell surface hydrophobicity. No direct correlation was found between adherence to hydrocarbons and the ability to metabolize hydrocarbons. For example, *Staphylococcus aureus*, a bacterium incapable of degrading hydrocarbons, adhered strongly to all test hydrocarbons, whereas *Pseudomonas aeruginosa* adhered poorly to hydrocarbons, despite its ability to grow on hydrocarbons under laboratory conditions.

The high affinity of *Acinetobacter calcoaceticus* RAG-1 towards liquid hydrocarbons enabled the isolation of a spontaneous nonadherent mutant, MR-481. Strain MR-481 exhibited no significant affinity toward the three test hydrocarbons yet resembled the wild type in many properties, including production of the extracellular emulsifying agent, emulsan. In order to study more closely the role of adherence in growth on hydrocarbons, RAG-1 and MR-481 were compared for growth on hexadecane under conditions of limited agitation and at low initial cell density. Adherent RAG-1 cells were able to grow rapidly under these conditions, whereas nonadherent MR-481 cells failed to grow for at least 54 hr. However, addition of emulsan either initially or at various times after inoculation enabled the nonadherent MR-481 cells to grow on hexadecane. Growth was not the result of reversion of MR-481 from nonadherent to adherent cells.

The data demonstrate that adherence is a crucial factor in the growth of *A. calcoaceticus* RAG-1 on hexadecane in the absence of extracellular emulsification of the substrate.

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REFERENCES

1 SHAPIRO, L. 1976. Differentiation in the caulobacter cell cycle. Ann. Rev. Microbiol. *30*:377–407.

2 STARR, M.P., and J.C.C. HUANG. 1972. Physiology of the Bdellovibrios. *In* A.H. Rose and D.W. Tempest (ed.), Advances in microbial physiology. *8*:215–261. Academic Press, London.

3 VARON, M., and M. SHILO. 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *B. bacteriovorus*. J. Bacteriol. *95*:744–753.

4 DWORKIN, M. 1973. Cell-to-cell interactions in the myxobacteria. Symp. Soc. Gen. Microbiol. *23*:125–142.

5 STANIER, R.Y. 1942. The cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. *6*:143-196.

6 OFEK, I., and E.H. BEACHEY. 1980. General concepts and principles of bacterial adherence in animals and man, p. 1–31. *In* E.H. Beachey (ed.), Bacterial adherence. Chapman and Hall, London and New York.

7 LIPPINCOTT, J.A., and B. LIPPINCOTT. 1980. Microbial adherence in plants, p. 375–398. *In* E.H. Beachey, (ed.), Bacterial adherence. Chapman and Hall, New York.

8 DANIELS, S.L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces, p. 7–58. *In* G. Bitton and K.C. Marshall (ed.), Adsorption of microorganisms to surfaces. John Wiley & Sons, New York.

9 MARSHALL, K.C. 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass.

10 LATHAM, M.J. 1980. Adhesion of rumen bacteria to plant cell walls, p. 339–350. *In* R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.

11 STANIER, op cit.

12 SUNDARRJ, C.O., and J.V. BHAT. 1972. Breakdown of chitin by *Cytophaga johnsoni*. Arch. fur Mikrobiologie *85*:159-167.

13 GIBSON, D.T. 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. *1*:199–223.

14 RATLEDGE, C. 1978. Degradation of aliphatic hydrocarbons, p. 1–46. *In* R.J. Watkinson (ed.), Developments in biodegradation of hydrocarbons. I. Applied Science Publishers, London.

15 FOSTER, J.W. 1962. Bacterial oxidation of hydrocarbons, p. 241–261. *In* O. Hayaishi (ed.), Oxygenases. Academic Press, New York.

16 KIRSCHNER ZILBER, I., E. ROSENBERG, and D. GUTNICK. 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on n-tetracosane. Appl. Environ. Microbiol. *40*:1086–1093.

17 NAKAHARA, T., L.E. ERICKSON, and J.R. GUTIERREZ. 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotech. Bioeng. *19*:9–25.

18 BLANCHARD, D.C., and L.D. SYZDEK. 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. *23*:389–400.

19 MARSHALL, op cit.

20 DEXTER, S.C., J.D. SULLIVAN JR., J. WILLIAMS III, and S.W. WATSON. 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl. Microbiol. *30*:298–308.

21 FLETCHER, M., and G.I. LOEB. 1979. The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. *37*:67–72.

22 GERSON, D.F., and D. SCHEER. 1980. Cell surface energy, contact angles and phase partition. III. Adhesion of bacterial cells to hydrophobic surfaces. Biochem. Biophys. Acta *602*:506–510.

23 ROSENBERG, M. 1981. Bacterial adherence to polystryene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. *42*:375–377.

24 BEACHEY, E.H., W.A. SIMPSON, and I. OFEK. 1980. Interaction of surface polymers of *Streptococcus pyogenes* with animal cells, p. 389–405. *In* R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.

25 PERERS, L., L. ANDÄKER, L. EDEBO, O. STENDAHL, and C. TAGESSON. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta. Path. Microbiol. Scand. Sect. B *85*:308–316.

26 ROSENBERG, M., A. PERRY, E.A. BAYER, D.L. GUTNICK, E. ROSENBERG, and I. OFEK, 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. *33*:29–33.

27 SMYTH, C.J., P. JONSSON, E. OLSSON, O. SÖDERLIND, J. ROSENGREN, S. HJERTÉN, and T. WADSTRÖM. 1978. Differences in hydrophobic surface characteristics of procine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. *22*:462–472.

28 VAN OSS, C.J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. *32*:19–39.

29 ROSENBERG, M., D. GUTNICK, and E. ROSENBERG. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. *9*:29–33.

30 ROSENBERG, M., E. ROSENBERG, and D. GUTNICK. 1980. Bacterial adherence to hydrocarbons, p. 541–542. *In* R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.

31 ROSENBERG, E., K.H. KELLER, and M. DWORKIN 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. J. Bacteriol. *129*:770–777.

32 OFEK and BEACHEY, op cit.

33 ROSENBERG, E., A. ZUCKERBERG, C. RUBINOWITZ, and D.L. GUTNICK 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. *37*:402–408.

34 ZUCKERBERG, A., A. DIVER, Z. PEERI, D.L. GUTNICK, and E. ROSENBERG. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. *37*:414–420.

35 ROSENBERG, GUTNICK, and ROSENBERG, op cit.

36 ROSENBERG, PERRY, BAYER, GUTNICK, ROSENBERG, and OFEK, op cit.

37 ROSENBERG, ROSENBERG, and GUTNICK, op cit.

38 PINES, O., and D.L. GUTNICK. 1981. Relationship between phase resistance–emulsan production and interaction of phages with the cell surface of *Acinetobacter calcoaceticus* RAG-1. Arch. Microbiol.

39 NAKAHARA, ERICKSON, and GUTIERREZ, op cit.

40 BELSKY, I., D.L. GUTNICK, and E. ROSENBERG. 1979. Emulsifier of *Arthrobacter* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. *101*:175–178.

41 REISFELD, A., E. ROSENBERG, and D. GUTNICK 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Environ. Microbiol. *24*:363–368.

42 ROSENBERG, ZACKERBERG, RUBINOWITZ, and GUTNICK, op cit.

43 ZUCKERBERG, DIVER, PEERI, GUTNICK, and ROSENBERG, op cit.

44 MUDD, S., and E.B.H. MUDD. 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. *40*:633–645.

45 GUTNICK, D.L., and E. ROSENBERG. 1977. Oil tankers and pollution: a microbiological approach. Ann. Rev. Microbiol. *31*:379–396.

46 KIRSCHNER ZILBER, ROSENBERG, and GUTNICK, op cit.

47 MARSHALL, op cit.

48 McLEE, A.G., and S.L. DAVIES. 1972. Linear growth of a *Torulopsis* sp. on n-alkanes. Can. J. Microbiol. *18*:315–319.

49 NAKAHARA, ERICKSON, and GUTIERREZ, op cit.

50 NEUFELD, R.J., J.E. ZAJIC, and D.F. GERSON. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. Appl. Environ. Microbiol. *39*:511–517.

51 CHAKRAVARTY, M., H.D. SINGH, and J.N. BARUAH. 1975. A kinetic model for microbial growth on liquid hydrocarbons. Biotech. Bioeng. *17*:399–412.

52 VELANKAR, S.K., S.M. BARNETT, C.W. HOUSTON, and A.R. THOMPSON. 1975. Microbial growth on hydrocarbons–some experimental results. Biotech. Bioeng. *17*:241–251.

53 WANG, D.I.C., and A. OCHOA. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentation and relationships to specific growth rates. Biotech. Bioeng. *14*:345–360.

54 ROSENBERG, GUTNICK, and ROSENBERG, op cit.

55 ROSENBERG, PERRY, BAYER, GUTNICK, ROSENBERG, and OFEK, op cit.

56 ROSENBERG, ROSENBERG, and GUTNICK, op cit.

57 BELSKY, GUTNICK, and ROSENBERG, op cit.

58 REISFELD, ROSENBERG, and GUTNICK, op cit.

59 ROSENBERG, ZUCKERBERG, RUBINOWITZ, and GUTNICK, op cit.

60 ZUCKERBERG, DIVER, PEERI, GUTNICK, and ROSENBERG, op cit.

61 LANKFORD, C.E., J.R. WALKER, J.B. REEVES, N.H. NABBUT, B.R. BYERS, and R.J. JONES. 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s). ("Schizokinen"). J. Bacteriol. *91*:1070–1079.

62 ROSENBERG, KELLER, and DWORKEN, op cit.

63 ROSENBERG, M., and E. ROSENBERG. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. *148*: 51–57.

64 CHAKRAVARTY, SINGH, and BARUAH, op cit.

65 HISATSUKA, K., T. NAKAHARA, Y. MINODA, and K. YAMADA. 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agr. Biol. Chem. *39*:999–1005.

66 VALENKAR, BARNETT, HOUSTON, and THOMPSON, op cit.

67 WANG and OCHOA, op cit.

68 MUDD and MUDD, op cit.

69 ROSENBERG, GUTNICK, and ROSENBERG, op cit.

70 BEACHEY, SIMPSON, and OFEK, op cit.

71 BLANCHARD and SYZDEK, op cit.

72 VAN OSS, op cit.

73 BEACHEY, SIMPSON, and OFEK, op cit.

74 MARSHALL, op cit.

75 OFEK and BEACHEY, op cit.

76 ROSENBERG and ROSENBERG, op cit.

ADDITIONAL REFERENCES

BAYER, E.A., E. ROSENBERG, and D. GUTNICK. 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. J. Gen Microbiol.

Adherence of Bacteria to Hydrocarbons

E. Rosenberg, M. Rosenberg, and D. L. Gutnick
Department of Microbiology
George S. Wise Faculty of Life Sciences
Tel Aviv University
Ramat Aviv, Israel

ABSTRACT

Two general types of interaction between microorganisms and hydrocarbons have been postulated: (A) adherence of cells directly to large oil droplets and (B) release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon:aqueous interfacial area. In the case of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 both types of interaction are operative; cells adhere avidly to test hydrocarbons (xylene, octane, hexadecane and crude oil) and also produce a potent polyanionic emulsifier referred to as emulsan. Mutants of *A. calcoaceticus* RAG-1 deficient in emulsan synthesis are still able to adhere to hydrocarbons and grow on hexadecane or crude oil as the sole source of carbon and energy. However, mutants of *A. calcoaceticus* RAG-1 unable to adhere to hydrocarbons also failed to grow on hydrocarbon substrates.

It was demonstrated that adherence is a prerequisite for growth on hexadecane under two conditions: low initial cell density and limited emulsification of the substrate. Such conditions prevail in most natural environments. On the other hand, bioemulsification is a cell density dependent phenomenon. Relatively high cell densities are required to produce enough extracellular emulsifying agent to markedly affect the hydrocarbon substrate.

Adherence of microorganisms to hydrocarbons is neither an exclusive property of hydrocarbon-degrading microorganisms nor restricted to those hydrocarbons that the microorganism can metabolize. For example, *Staphylococcus aureus, Serratia marcescens* and *Streptococcus pyogenes* adhered avidly to test hydrocarbons as a result of their high cell surface hydrophobicity, but were unable to metabolize any of the hydrocarbon substrates tested. *A. calcoaceticus* RAG-1 can grow on alkanes, but not aromatics; however, it adhered equally well to both substances. Further, certain bacteria that have the genetic potential to degrade hydrocarbons, e.g., *Pseudomonas aeruginosa,* adhere poorly to hydrocarbons. It follows that introduction of hydrocarbon-degrading plasmids into microorganisms with low cell surface hydrophobicity may not lead to cells that interact well with hydrocarbons in open systems.

INTRODUCTION

The importance of cell adherence in growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces.[36] The initial step in the life cycle of *Bdellovibrio* is attachment to Gram-negative bacteria.[39,41] Specific cell-to-cell interactions pervade the entire life cycle of the myxobacteria.[8,38] Adhesion is a prerequisite to the successful colonization by microorganisms of animals[24] and plants.[18] Microbial adhesion to surfaces has been the subject of several recent reviews.[6,17,18,19,24]

In cases where the carbon or energy source is a water-insoluble material, such as cellulose, or chitin, bacterial adhesion can facilitate growth,[17,38,40] but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, growth of microorganisms on hydrocarbons presents a special problem since not only are hydrocarbons immiscible with water, but also their breakdown cannot occur extracellularly. The first step in aromatic[12] or aliphatic[27] hydrocarbon degradation is introduction of molecular oxygen into the molecules by cell-associated enzymes.[10]

Two general types of hydrocarbon-cell interactions have been postulated, depending upon the state and size of the oil droplets relative to the size of the microbial cells:[15] adherence of cells to large oil droplets and pseudosolublization involving the cellular assimilation of emulsified small hydrocarbon droplets. The relative contribution of these two types of interactions to the growth of bacteria on hydrocarbon has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon: aqueous interfacial area and growth rate can be used to support either hypothesis.[22]

Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which bacterial hydrophobicity and adherence to hydrophobic surfaces are involved. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in the partitioning of microorganisms at the air:water interface,[4,19] in adherence of bacteria to nonwettable plastic surfaces,[7,9,11,31] and in the attachment of bacteria to phagocytes[42] and other mammalian cells.[2,25,33,37]

We previously described a rapid and quantitative method for the measurement of bacterial adherence to hydrocarbons.[32,34] The present report deals with the use of this technique in (1) examining the cell surface hydrophobicity of various bacterial species, some of which do not metabolize hydrocarbons, and (2) selecting and isolating non-adherent mutants. The isolation of a non-adherent mutant of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 has enabled us to examine the role of adherence in bacterial growth on hexadecane.

1983

MATERIALS AND METHODS

Bacterial strains—Tables 1 and 2 contain a list of bacteria used in this study. *Acinetobacter calcoaceticus* MR-481 is a non-adhering derivative of strain RAG-1.

Media and growth conditions—For measuring adherence to test hydrocarbons or preparing inocula for growth experiments, 0.1 ml bacteria of an overnight culture were inoculated into 50 ml growth medium and incubated in a New Brunswick Model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested at the end of exponential phase, washed twice and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_3O$; 7.26 g KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$, and distilled water to 1000 ml, pH 7.1). Growth media were: BHI—brain heart infusion broth (Difco); hexadecane medium—PUM buffer supplemented with 0.2 percent (v/v) hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland). P-hexadecane medium—5 g NH_4NO_3 , 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 ml hexadecane, 50 mg threonine and tap-water to 1000 ml; NB—nutrient broth (Difco) supplemented with 0.5 percent NaCl. CT medium contained 1 percent casitone (Difco) and 0.2 percent $MgSO_4 \cdot 7H_2O$.[29]

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14 mm diameter) containing 4.2 ml aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2 percent sodium acetate. Hexadecane growth medium consisted of 0.5 ml hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick gyrotory water bath shaker (Model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Adherence to hydrocarbons—The technique for measuring adherence of bacteria to hydrocarbons has been described previously.[24] Cells were washed twice and resuspended in PUM buffer to an initial A_{400} of approximately 1.5. Various volumes of the test hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed cell suspension and the phases were vortexed uniformly for 120 sec. After allowing 15 min for the phases to separate, the absorbence of the lower aqueous phase of each tube was measured at 400 nm (Gilford model 240 spectrophotometer, 1 cm light path). Adherence was calculated as the percentage loss in absorbence relative to that of the initial cell suspension.

Isolation of the non-adherent mutant MR-481—A spontaneous mutant of RAG-1 which is deficient in its ability to adhere to hydrocarbons was isolated by the following technique: RAG-1 cells were inoculated into 50 ml nutrient broth medium (NB) and incubated in a New Brunswick model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested in stationary phase following 18 hr growth, washed twice and resuspended in PUM buffer to an A_{400} of approximately 1.5. To 5 ml of the cell suspension were added 2 ml of n-octane, and the phases vortexed for 5 min. Following phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases were mixed as described previously. This extraction process was continued until cells in the lower aqueous phase were too few to be visible under microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to stationary phase as previously described. Cells were again harvested, washed and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. The final aqueous phase was plated on nutrient agar, and three individual colony types were isolated and tested for adherence to hydrocarbons. One clone of cells, termed MR-481, exhibited no significant affinity towards the test hydrocarbons, and was chosen for further study.

Emulsan preparation—The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously.[30] The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH Chem. Ltd.), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing extensively against distilled water. The material was then deproteinized by the phenol method[45] and dialyzed. The final product had a reduced viscosity of 490 cc per g, an emulsifying activity of 150 units per mg, an ester content of 0.5 μmoles per mg and contained less than 0.5 percent protein. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and sterilized by autoclave prior to its addition in the growth experiments.

RESULTS

Bacterial adherence to hydrocarbons—The basic assay for bacterial adherence t hydrocarbons is illustrated in Fig. 1.

The assay was performed with varying volumes of 3 test hydrocarbons, n-hexadecane, n-octane and p-xylene. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbons (e.g. *E. coli*), the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested (e.g., *A. calcoaceticus* RAG-1), a 'creamy' upper layer was obtained following mixing.

Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was found to be a function of volume of hydrocarbon added. The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5 percent isopropanol resulted in immediate droplet coalescence and the concomitant release of extracted cells into the aqueous phase, thereby demonstrating that the bacteria had partitioned to the hydrocarbon:water interface.

Tables 1 and 2 summarize a number of experiments in which cell surface hydrophobicity of a variety of bacteria was measured by adherence to hexadecane. The majority of laboratory strains examined exhibited low affinity towards the test hydrocarbon. Among the bacterial strains exhibiting high affinity for hexadecane were hydrocarbon-degrading *Acinetobacter calcoaceticus* strains RAG-1, HO1-N, B5W str 71 and CDC 7828, and bacteria which do not metabolize hydrocarbons: *Serratia marcescens, Staphylococcus aureus* and *Streptococcus pyogenes.*

Pseudomonas aeruginosa (which can utilize hexadecane as sole carbon and energy source) exhibited no significant affinity towards any of the test hydrocarbons when grown to late exponential phase in nutrient broth (Fig. 2). Since adherence in this organism might have required the presence of hydrocarbon during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane (Fig. 2). The affinity of alkane-grown *P. aeruginosa* towards hexadecane did increase somewhat (e.g., 32 percent of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane). In contrast, *A. calcoaceticus* RAG-1 exhibited much higher affinity when grown on hexadecane (93 percent of the cells could be removed by as little as 0.05 ml hexadecane).

Role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane

Acinetobacter calcoaceticus RAG-1 adheres avidly to hydrocarbons.[32,34] A spontaneous mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells which did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Fig. 3).

Mutant MR-481 resembled the wild type in the following characteristics: (a) colonial and cell morphology, (b) sensitivity to two bacteriophages, ap2 and ap3[26] which are specific for *A. calcoaceticus* RAG-1 strains, (c) production of extracellular emulsifying activity during growth on ethanol as sole carbon and energy source,[22] (d) doubling time on brain heart infusion broth (38 min at 30°C with aeration) and (e) agglutinability by antibodies raised against RAG-1 cells.[1]

TABLE 1—Adherence of Bacteria to Hexadecane

Strain	Source	Growth Medium[a]	Adherence to Hexadecane, pct[b]
Acinetobacter calcoaceticus RAG-1	This laboratory	BHI	97
Bacillus cereus	Local isolate	NB	1
Bacillus subtilis 168	E. Z. Ron	NB	0
Enterobacter aerogenes CDC $659/_{66}$	E. Z. Ron	NB	9
Escherichia coli B	E. Z. Ron	NB	0
Escherichia coli K12	E. Z. Ron	NB	2
Escherichia coli J5 (rough)	E. Z. Ron	NB	11
Micrococcus lysodeikticus (ATCC 4698)	I. Friedberg	NB	0
Myxococcus xanthus	This laboratory	CT	35
Proteus mirabilis S1959	S. Rottem	NB	0
Pseudomonas aeruginosa PAS279	J. Shapiro	NB	0
Serratia marcescens	R. Zack	NB	96
Staphlococcus albus	R. Zack	NB	0
Staphlococcus aureus	R. Zack	NB	91
Streptococcus pyogenes M5	I. Ofek	BHI	84

[a] Cells were harvested at the end of the exponential phase, washed twice and suspended in PUM buffer to an initial absorbence at 400 nm of 1.1-1.6.

[b] Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

TABLE 2—Adherence of *Acinetobacter* Strains to Hexadecane

Strain Designation	Source	Growth on Hexadecane[a]	Adherence to Hexadecane, pct[b]
RAG-1 (ATCC 31012)	This laboratory	+	82
HO1-N	W. R. Finnerty	+	99
BD4	E. Juni	+	4
BD413	E. Juni	+	0
B5W str 71 (ATCC 15149)	K. Bryn	+	84
CDC 7827 (ATCC 17959)	K. Bryn	+	86
B5W (ATCC 17903)	K. Bryn	−	0
ATCC 17906	K. Bryn	−	52
ATCC 17977	K. Bryn	−	7
ATCC 17985	K. Bryn	−	41

[a] Growth was examined on PUM agar in the presence of hexadecane vapors.

[b] Adherence was measured on cells grown to stationary phase on BHI broth; the standard adherence assay was performed with 0.05 ml hexadecane.

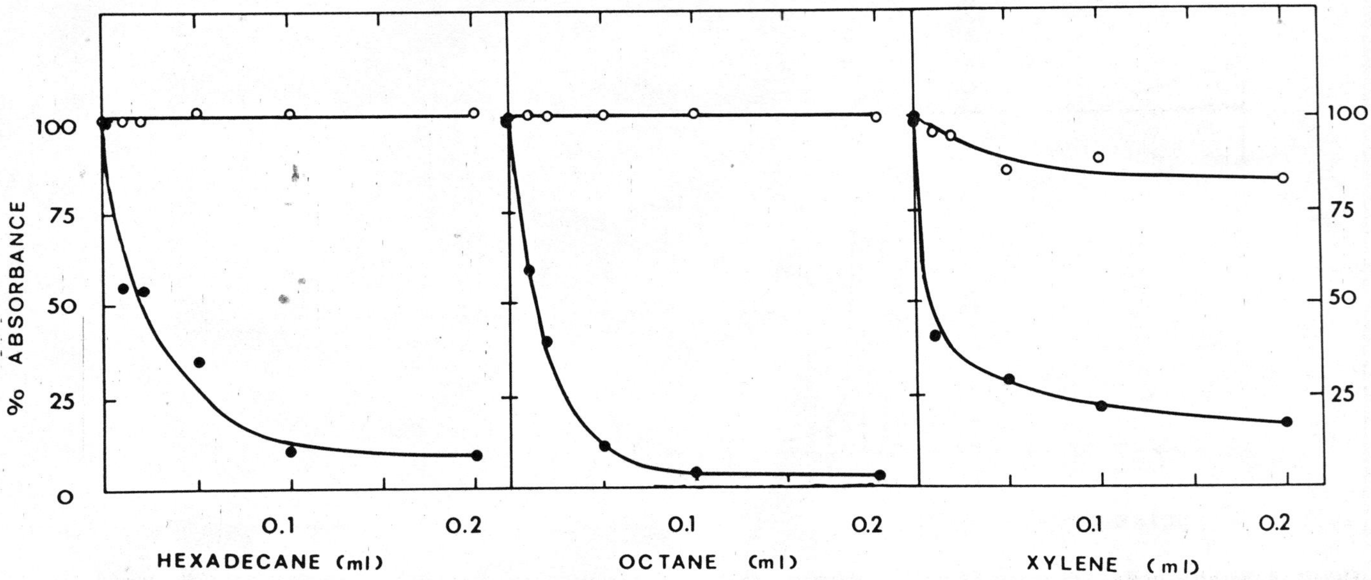

FIG. 1—Adherence of *Eschericia coli* B (○) and *Acinetobacter calcoaceticus* RAG-1 (•) to hydrocarbons. Cells were harvested at the end of the exponential growth phase, washed twice in PUM buffer and then resuspended to an initial A_{400} of 1.2-1.4. The adherence assay was performed as described in Materials and Methods. The ordinate is the percentage of initial absorbence of the cell suspension.

It should be emphasized that in all growth experiments inocula were prepared in the same manner as was described for measuring adherence in Fig. 3. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 4). Following a 2 hr lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew whereas the non-adherent mutant MR-481 failed to grow for at least 54 hr (Fig. 5). Growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells.

Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since attached bacteria were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Fig. 6).

Effect of emulsan on the growth of MR-481 on hexadecane

A. calcoaceticus RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan.[3,28,30,45] It was of interest to examine the effect of a highly purified preparation of emulsan (see Materials and Methods) on the growth of RAG-1 and the non-adherent mutant MR-481 on hexadecane (Fig. 7). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481.

However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both

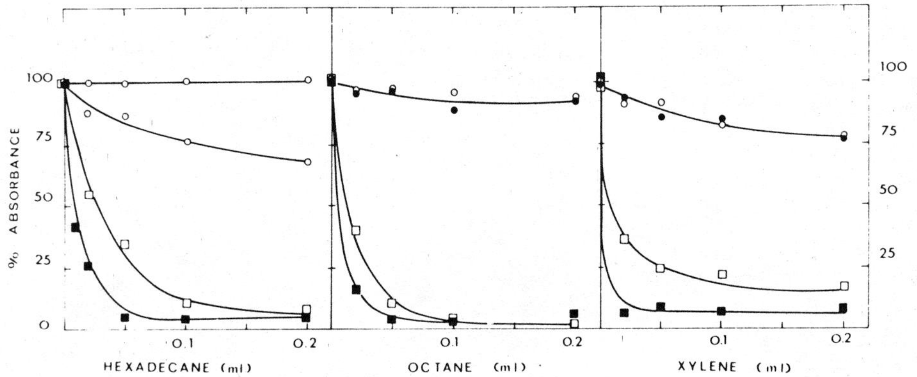

FIG. 2—Adherence of *Psuedomonas aeruginosa* PAS 279 and *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons. The experiment was performed as described in Fig. 1 with cells harvested at the end of the exponential growth phase: *P. aeruginosa*, NB medium (○), P-hexadecane medium (•); *A. calcoaceticus*, NB medium (□), hexadecane medium (■).

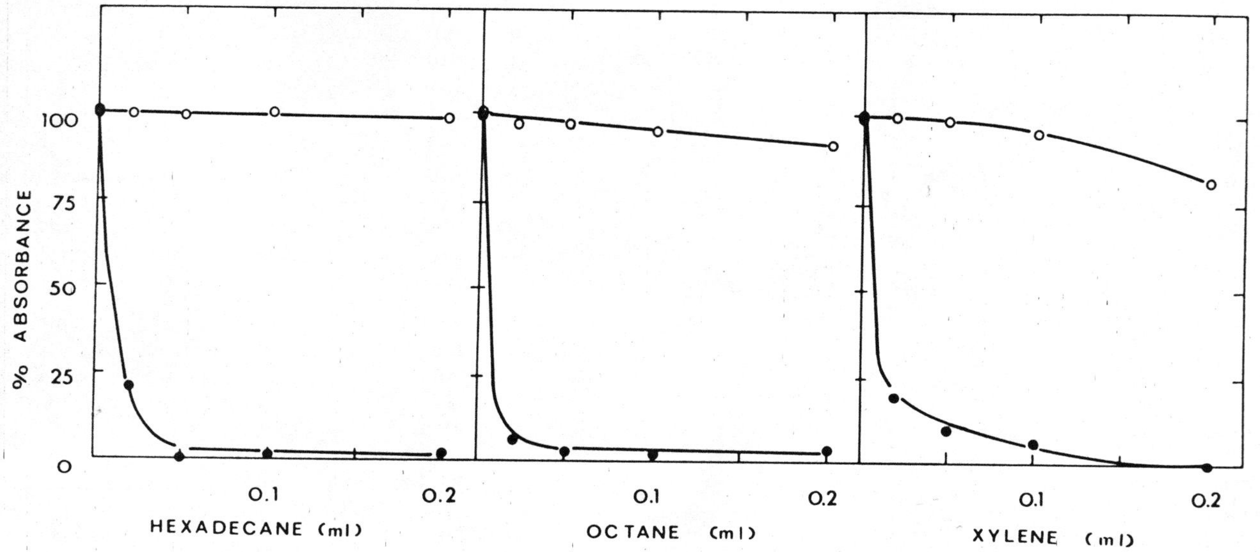

FIG. 3—Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth washed twice and resuspended in PUM buffer to an A_{400} = 1.45 to 1.50. To 1.2 ml of suspended RAG-1 (•) or MR-481 (○) cells were added to various volumes of test hydrocarbon and the mixtures vortexed for 120 sec. Following phase separation the turbidity of the lower aqueous phase was measured. The ordinate is the percent of initial absorbence of the cell suspension.

emulsan and hexadecane began after about 6 hr, whereas neither component alone supported growth for 54 hr. At the weight ratio of hexadecane: emulsan used, 1,925:1, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Fig. 8). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic or phenotypic

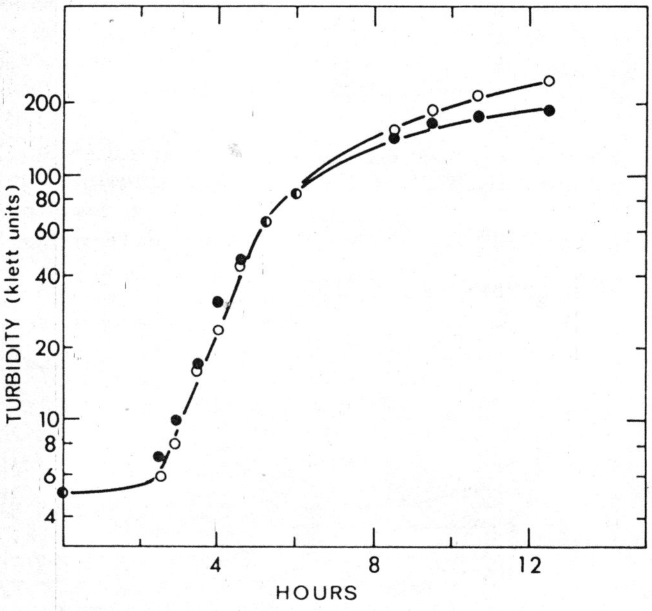

FIG. 4—Growth of RAG-1 and MR-481 in acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice and resuspended in PUM buffer supplemented with 0.2 percent sodium acetate. RAG-1 (•) and MR-481 (○) cells were inoculated into 4.2 ml acetate medium in Klett tubes to an initial turbidity of 5 Klett units.

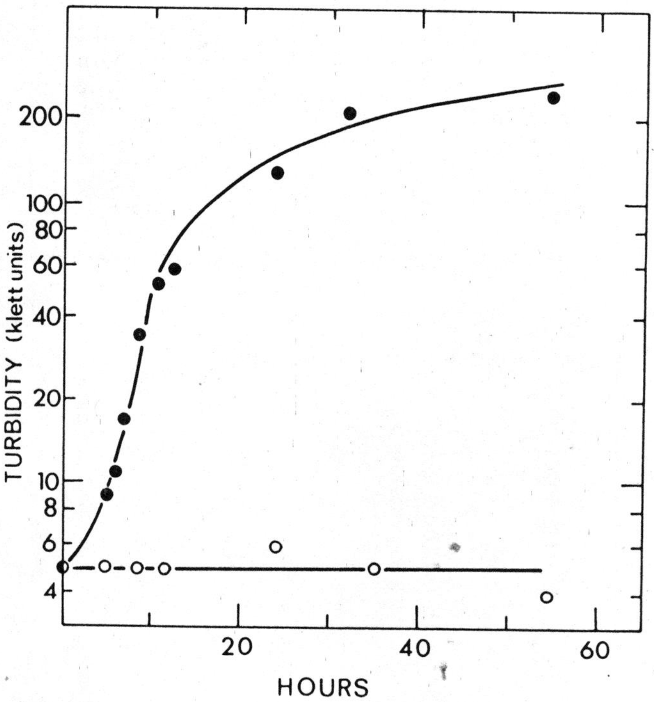

FIG. 5—Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml PUM buffer were inoculated with either RAG-1 (•) or MR-481 (○) to an initial turbidity of 5 Klett units. Tubes were overlayed with 0.5 ml hexadecane and incubated as described in Materials and Methods.

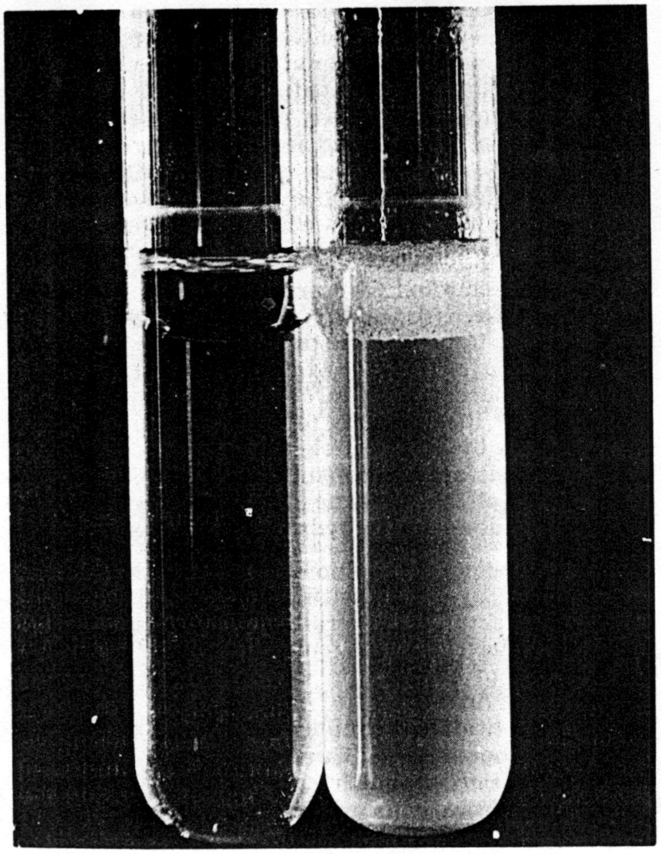

FIG. 6—Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. Tubes were photographed following 48 hr incubation under the conditions described in Fig. 3.

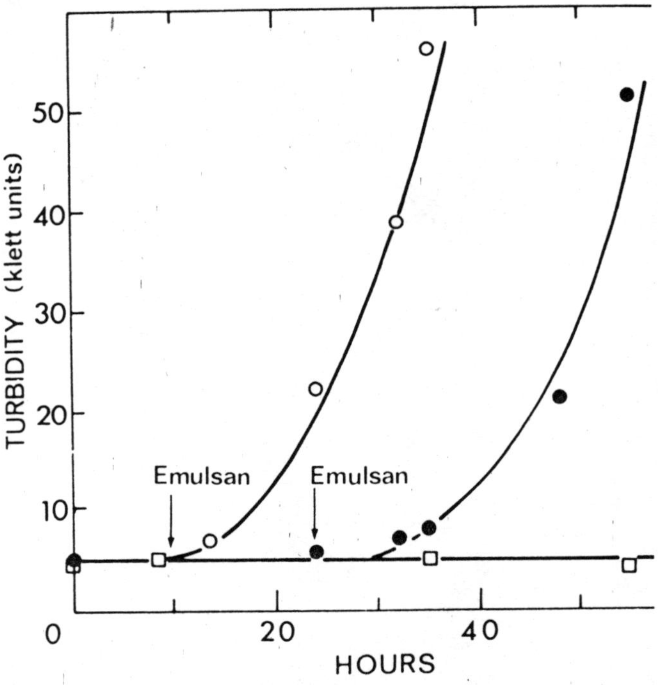

FIG. 8—Induction of growth of MR-481 on hexadecane by emulsan addition. Inocula preparation and incubation conditions were as described in Fig. 3. Emulsan was added to a final concentration of 50 μg/ml at 10 hr (○) or 24 hr (●). Tubes containing no emulsan served as controls (□).

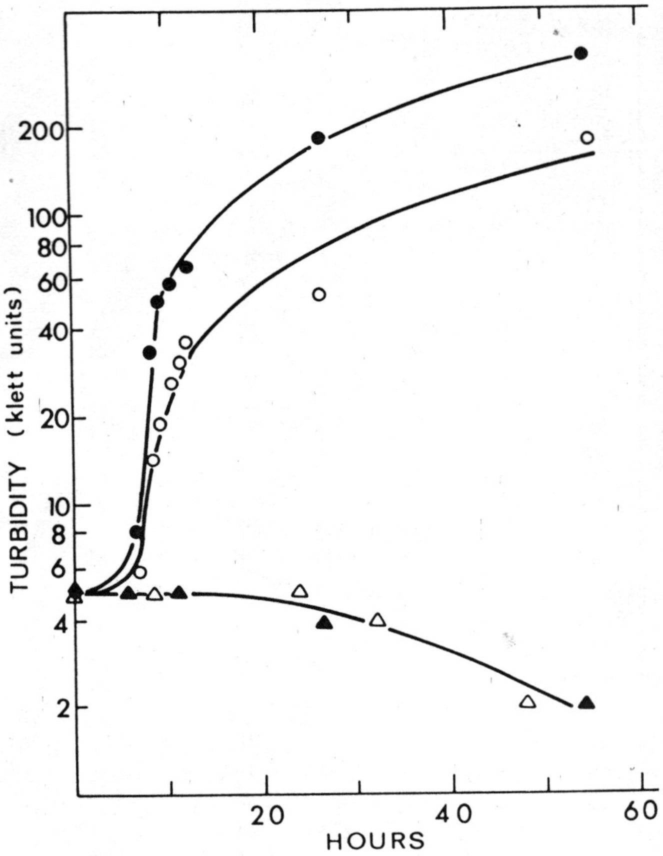

FIG. 7—Effect of emulsan on the growth of MR-481 on hexadecane. Inocula preparation and incubation conditions were as described in Fig. 3. RAG-1 (●) and MR-481 (○) were grown on hexadecane medium in the presence of 50 μg/ml emulsan. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg/ml emulsan, in the absence of hexadecane.

reversion of MR-481 from non-adherent to adherent cells. As shown in Table 3, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared to the initial inoculum grown in rich medium (Fig. 3).

RAG-1 cells which were harvested and washed following growth on hexadecane both in the presence and absence of emulsan, exhibited high affinity towards hexadecane (Table 3). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

DISCUSSION

Mudd and Mudd[21] reported over 50 years ago that certain microorganisms tend to concentrate at oil:water interfaces. Although this observation has been frequently corroborated during studies on the growth of microorganisms on liquid hydrocarbons,[13,15,19,20,22,23] there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Certain investigators have argued that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets.[5,43,44] *A. calcoaceticus* RAG-1 is an interesting microorganism in

TABLE 3—Adherence of RAG-1 and MR-481 Following Growth on Hexadecane

Strain	Turbidity (A_{400})[a]		Percent Adherence
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR-481[c]	1.43	1.36	4.9

[a] Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested following 54 hr growth on hexadecane medium, washed twice and resuspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested following 54 hr growth on hexadecane medium in the presence of 50 μg/ml emulsan, washed twice and resuspended in PUM buffer.

this regard because it both adheres avidly to hydrocarbons[32,33,34] and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized.[3,28,30,45]

Isolation of a mutant strain of *A. calcoaceticus* RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The non-adherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were excluded by the following data: (1) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (2) MR-481 was able to grow on hexadecane medium following addition of an emulsifier; and (3) MR-481 cells were able to grow on solid medium supplemented with hexadecane vapors as sole carbon and energy source. We conclude from these data that adherence is a prerequisite for growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce a potent extracellular emulsifying agent which enables growth of non-adhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon.[16,29,35] At the low inoculum employed (5 × 10^7 cells/ml) the concentration of emulsan which the cells produce is too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of non-adherent bacteria on hydrocarbons. The poor adherence of *P. aeruginosa* PAS 279 to hydrocarbon (Fig. 2) suggests that its growth, similar to that of MR-481, depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments, conditions of low cell density and limited emulsification prevail; thus, the growth of poorly-adhering *pseudomonas* strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation[5,14,43,44] probably does not reflect growth in the open environment, where emulsifier and dispersed hydrocarbon would tend to diffuse away from the cell.

The adherence to hydrocarbon of bacteria which do not metabolize hydrocarbons (Tables 1 and 2) can be explained in terms of general cell surface hydrophobicity.[21,32] *S. pyogenes,* for example, has been reported to adhere to buccal epithelial cells through hydrophobic interactions.[2] Its adherence to hydrocarbons may thus be mediated by such interactions. Similarly, hydrophobic cell surface components which enable *Serratia* to partition at the air:water interface[4] may also promote their adherence to hydrocarbons as observed in this study.

Bacterial hydrophobicity has been cited as an important factor in several biological phenomena, including bacterial pathogenicity,[42] and in adherence and growth of bacteria on various hydrophobic surfaces.[2,19,24,35] Adherence to hydrocarbons provides a simple quantitative technique for measuring cell surface hydrophobicity and can be used for the selection and isolation of non-adhering mutants. Adherence of RAG-1 to hydrocarbons may reflect a general phenomenon in microbiology: A biological advantage is conferred on microbial cells which can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface which provides nutrition and enables growth.

REFERENCES

1. Bayer, E. A., E. Rosenberg and D. Gutnick. 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. J. Gen Microbiol. *127:* 295-300.

2. Beachey, E. H., W. A. Simpson and I. Ofek. 1980. Interaction of surface polymers of *Streptococcus pyogenes* with animal cells, p. 389-405. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.). Microbial Adhesion to Surfaces. Society of Chemical Industry, London.

3. Belsky, I., D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: Determination of emulsifier-bound fatty acids. FEBS Lett. *101:* 175-178.

4. Blanchard, D. C. and L. D. Syzdek. 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. *23:* 389-400.

5. Chakravarty, M., H. D. Singh and J. N. Baruah. 1975. A kinetic model for microbial growth on liquid hydrocarbons. Biotech. Bioeng. *17:* 399-412.

6. Daniels, S. L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces, pp. 7-58. *In* G. Bitton and K. C. Marshall (Eds.). Adsorption of Microorganisms to Surfaces. John Wiley & Sons, New York.

7. Dexter, S. C., J. D. Sullivan, Jr., J. Williams, III, and S. W. Watson. 1975. Influence of substrate wettabil-

ity on the attachment of marine bacteria to various surfaces. Appl. Microbiol. *30:* 298-308.

8. Dworkin, M. 1973. Cell-to-cell interactions in the myxobacteria. Symp. Soc. Gen. Microbiol. *23:* 125-142.

9. Fletcher, M. and G. I. Loeb. 1979. The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. *37:* 67-72.

10. Foster, J. W. 1962. Bacterial oxidation of hydrocarbons, pp. 241-261. *In:* Oxygenases. O. Hayaishi (Ed.), Academic Press, New York.

11. Gersen, D. F. and D. Sheer. 1980. Cell surface energy, contact angles and phase partition. III Adhesion of bacterial cells to hydrophobic surfaces. Biochem. Biophys. Acta *602:* 506-510.

12. Gibson, D. T. 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. *I:* 199-223.

13. Gutnick, D. L. and E. Rosenberg. 1977. Oil tankers and pollution: A microbiological approach. Ann. Rev. Microbiol. *31:* 379-396.

14. Hisatsuka, K., T. Nakahara, Y. Minoda and K. Yamada. 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agr. Biol. Chem. *39:* 999-1005.

15. Kirschner Zilber, I., E. Rosenberg and D. Gutnick. 1980. Incorporation of ^{32}P and growth of pseudomonad UP-2 on n-tetracosane. Appl. Environ. Microbiol. *40:* 1086-1093.

16. Lankford, C. E., J. R. Walker, J. B. Reeves, N. H. Nabbut, B. R. Byers and R. J. Jones. 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s). ("Schizokinen"). J. Bacteriol. *91:* 1070-1079.

17. Latham, M. J. 1980. Adhesion of rumen bacteria to plant cell walls, p. 339-350. *In:* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (Eds.), Microbial Adhesion to Surfaces. Society of Chemical Industry.

18. Lippincott, J. A. and B. Lippincott. 1980. Microbial adherence in plants, p. 375-398. *In:* E. H. Beachey, (Ed.), Bacterial Adherence. Chapman and Hall, New York.

19. Marshall, K. C. 1976. Interfaces in Microbial Ecology. Harvard University Press. Cambridge, Mass.

20. McLee, A. G. and S. L. Davies. 1972. Linear growth of a *Torulopsis* sp. on n-alkanes. Can. J. Microbiol. *18:* 315-319.

21. Mudd, S. and E. B. H. Mudd. 1924. The penetration of bacteria through capilary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. *40:* 633-645.

22. Nakahara, T., L. E. Erickson and J. R. Gutierrez. 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotech Bioeng. *19:* 9-25.

23. Neufeld, R. J., J. E. Zajic and D. F. Gerson. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. Appl. Environ. Microbiol. *39:* 511-517.

24. Ofek, I. and E. H. Beachey. 1980. General concepts and principles of bacterial adherence in animals and man. p. 1-31. *In:* E. H. Beachey (Ed.). Bacterial Adherence, Chapman and Hall, London and New York.

25. Perers, L., L. Andaker, L. Edebo, O. Stendahl and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta. Path. Microbiol. Scand. Sect. B *85:* 308-316.

26. Pines, O. and D. L. Gutnick. 1981. Relationship between phage resistance—emulsan production and interaction of phages with the cell surface of *Acinetobacter calcoaceticus* RAG-1. Arch. Microbiol. *130:* 129-133.

27. Ratledge, C. 1978. Degradation of aliphatic hydrocarbons, p. 1-46. *In:* R. J. Watkinson (Ed.). Developments in Biodegradation of Hydrocarbons—I. Applied Science Publishers, London.

28. Reisfeld, A., E. Rosenberg and D. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Environ. Microbiol. *24:* 363-368.

29. Rosenberg, E., K. H. Keller and M. Dworkin. 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. J. Bacteriol. *129:* 770-777.

30. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D. L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. *37:* 402-408.

31. Rosenberg, M. 1981. Bacterial adherence to polystryene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. *42:* 375-377.

32. Rosenberg, M., D. Gutnick and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. *9:* 29-33.

33. Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg and I. Ofek. 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. *33:* 29-33.

34. Rosenberg, M., E. Rosenberg and D. Gutnick. 1980. Bacterial adherence to hydrocarbons, p. 541-542. *In:* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.). Microbial Adhesion to Surfaces. Society of Chemical Industry, London.

35. Rosenberg, M. and E. Rosenberg. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. *148:* 51-57.

36. Shapiro, L. 1976. Differentiation in the caulobacter cell cycle. Ann. Rev. Microbiol. *30:* 377-407.

37. Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén and T. Wadström. 1978. Differences in hydrophobic surface characteristics of procine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. *22:* 462-472.

38. Stanier, R. Y. 1942. The cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. *6:* 143-196.

39. Starr, M. P. and J. C. C. Huang. 1972. Physiology of the *Bdellovibrios*. *In:* Advances of Microbiological Physiology. *8:* 21-261. A. H. Rose and D. W. Tempest (Eds.). Academic Press, London.

40. Sundarrj, C. P. and J. V. Bhat. 1972. Breakdown of chitin by *Cytophaga johnsoni*. Arch. fur Mikrobiologie *85:* 159-167.

41. Varon, M. and M. Shilo. 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *B. Bacteriovorus*. J. Bacteriol. *95:* 744-753.

42. van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. *32:* 19-39.

43. Velankar, S. K., S. M. Barnett, C. W. Houston and A. R. Thompson. 1975. Microbial growth on hydrocarbons—some experimental results. Biotech. Bioeng. *17:* 241-251.

44. Wang, D. I. C. and A. Ochoa. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentation and relationships to specific growth rates. Biotech. Bioeng. *14:* 345-360.

45. Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. *37:* 414-420.

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Bacterial Degradation of Emulsan

YUVAL SHOHAM, MEL ROSENBERG,† and EUGENE ROSENBERG*

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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Emulsan is a polyanionic heteropolysaccharide bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1. A mixed bacterial population was obtained by enrichment culture that was capable of degrading emulsan and using it as a carbon source. From this mixed culture, an emulsan-degrading bacterium, termed YUV-1, was isolated. Strain YUV-1 is an aerobic, gram-negative, non-spore-forming, rod-shaped bacterium which grows best in media containing yeast extract. When placed on preformed lawns of *A. calcoaceticus* RAG-1, strain YUV-1 produced translucent plaques which grew in size until the entire plate was covered. Plaque formation was due to solubilization of the emulsan capsule of RAG-1. Plaque formation was not observed on emulsan-negative mutants of RAG-1. As a consequence of the solubilization of the emulsan capsule, RAG-1 cells became more hydrophobic, as determined by adherence to hexadecane. Growth of YUV-1 on a medium containing yeast extract and emulsan was biphasic. During the initial 24 h, cell concentration increased 10-fold, but emulsan was not degraded; during the lag in growth (24 to 48 h), emulsan was inactivated and depolymerized but not consumed; during the second growth phase (48 to 70 h) the depolymerized emulsan products were consumed.

In recent years, bioemulsifiers have received increasing attention both from the point of view of the growth physiology of the microorganisms which produce them (9, 14–16, 18, 39) and from that of their potential commercial exploitation (5, 11, 40). Emulsan, the extracellular emulsifier of *Acinetobacter calcoaceticus* RAG-1 (27, 29), stabilizes a wide variety of hydrocarbon-in-water emulsions by forming a strong polymeric film on the interface (28, 41). The amphipathic nature of emulsan is probably due to the presence of a hydrophilic heteropolysaccharide (D-galactosamine, aminouronic acid, and a third unidentified amino sugar) and hydrophobic fatty acids (12 to 15%) joined to the backbone through *O*-ester and *N*-acyl linkages (3, 42).

As is the case with many other extracellular polysaccharides (36), emulsan is produced largely after the cells reach stationary phase (10, 29). Recently, evidence has been presented that during exponential growth, emulsan accumulates on the cell surface of RAG-1 as a mini-capsule (10, 26, 32; O. Pines, E. Bayer, and D. L. Gutnick, submitted for publication).

For ecological considerations, the stability of the bioemulsifier to microbial degradation is an important factor in selecting appropriate applications. In this paper, we report studies on depolymerization and utilization of emulsan by mixed and pure bacterial cultures. In addition, evidence is presented that an emulsan-degrading bacterium, strain YUV-1, can solubilize the emulsan capsule of RAG-1.

MATERIALS AND METHODS

Bacterial strains. *A. calcoaceticus* RAG-1 (ATCC 31012) was initially isolated after enrichment on crude oil medium (27). *A. calcoaceticus* AG-1, TR3, and 10 are emulsan-negative mutants of RAG-1 (26). *A. calcoaceticus* BD4 is a heavily encapsulated (rhamnose-glucose polymer) strain (17, 37), kindly provided by K. Bryn. Strain YUV-1 is an emulsan-degrading bacterium isolated during the course of this study. All *A. calcoaceticus* strains were maintained on Luria broth (LB) agar and subcultured monthly. Strain YUV-1 was maintained on LB agar and subcultured weekly.

Media and growth conditions. Minimal salts solution (MS buffer) contained, per liter of water: $K_2HPO_4 \cdot 3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; $(NH_4)_2SO_4$, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g. The final pH was 7.2. Unless stated otherwise, broth medium consisted of MS buffer supplemented with 0.1% carbon source. When glucose was used, it was autoclaved separately.

For growth experiments, bacteria were cultivated in flasks on a New Brunswick G-53 Gyrotory shaker at 150 rpm at 30°C. Growth was monitored by measuring culture absorbance at 560 nm with a Gilford model 240 spectrophotometer.

Solid media were prepared by the addition of 1.5% Noble agar (Difco Laboratories) to liquid media. Ethanol agar was prepared by placing 0.2 ml of ethanol on Whatman 3MM filter paper and placing the filter on the petri dish cover of MS agar plates. The plates were incubated inverted.

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† Present address: School of Dental Medicine, Tel Aviv University, Ramat Aviv, Israel.

Enrichment culture procedure. A 5-g soil sample was inoculated into 20 ml of 1% yeast extract (Difco) medium and incubated at 30°C with shaking. After 12 h, 0.2 ml was transferred to 2 ml of MS buffer supplemented with 0.5% *N*-acetyl-D-galactosamine and incubated with shaking at 30°C for 24 h. Two more transfers were performed in the same galactosamine medium under the same conditions. A sample of the mixed culture (0.2 ml) was then inoculated into 2 ml of MS buffer supplemented with 0.1% emulsan and incubated with shaking. After 60 h, the culture was centrifuged at 12,100 × *g* for 10 min, the supernatant fluid was filtered through a 0.45-μm filter, and the viscosity of the cell-free filtrate was determined. Since the viscosity of the medium decreased sharply, the enrichment procedure on emulsan medium was continued. After three more transfers of the mixed culture in emulsan medium (1/10 dilution; 24 h of incubation), the resulting mixed culture was able to decrease the viscosity of the medium within 12 h. The mixed culture was maintained either by transferring into fresh emulsan medium every 3 days or by centrifuging the mixed culture and storing the frozen bacterial pellet at −21°C.

Emulsan. Emulsan, the polyanionic emulsifying agent of *A. calcoaceticus* RAG-1, was purified from the cell-free supernatant fluid obtained from an ethanol-grown culture (42). After dialysis and lyophilization, the powder was washed in warm ethanol to remove colored impurities and then extracted into 80% ethanol; emulsan was then precipitated by the addition of a saturated solution of $Ca(NO_3)_2$ in 90% ethanol. The extraction of emulsan into 80% ethanol and precipitation with $Ca(NO_3)_2$ was repeated. The product was then dissolved in water, dialyzed extensively against distilled water, and lyophilized. The emulsan preparation used in these studies had an emulsifying activity of 90 U/mg, an *O*-ester content of 0.5 μmol/mg, a reduced viscosity of 380 ml/g, and a residual protein content of 1.0% (see below).

Emulsification assay. Emulsan activity was determined by a standard emulsification assay (29) which is based on emulsification of a mixture of 0.1 ml of hexadecane–2-methylnaphthalene (1:1 [vol/vol]) in 7.5 ml of Tris-magnesium buffer (20 mM Tris-hydrochloride containing 10 mM $MgSO_4$ [pH 7.0]). After the mixture was shaken for 60 min at 30°C, the turbidity of the stable hydrocarbon-in-water emulsion was measured in a Klett-Summerson colorimeter. One unit of activity per milliliter of assay mixture is that amount of emulsan which gives rise to a turbidity of 100 Klett units in the standard emulsification assay.

A semiquantitative microassay for determining emulsan degradation by loss in emulsifying activity was also used. Samples (50 μl each) of emulsan media were added to round-bottom test tubes (8-mm diameter) containing 0.5 ml of standard Tris-magnesium buffer and 20 μl of the hexadecane-2-methylnaphthalene mixture (1:1 [vol/vol]). Tubes were vortexed on a Thermolyne Maxi Mix (Sybron Corp., Dubuque, Iowa) for 3 min. After the tubes were allowed to stand for 1 min, the emulsions were examined. Samples containing active emulsan formed stable emulsions; samples in which the emulsan had been inactivated separated into hydrocarbon and water phases.

Adherence to hexadecane. Adherence of bacteria to liquid hydrocarbons was measured by the method of Rosenberg et al. (31). Cells were washed twice and suspended in PUM buffer [MS buffer containing 1.8 g of urea per 1,000 ml instead of $(NH_4)_2SO_4$]. The absorbance at 400 nm of the cell suspension was adjusted to 1.4 to 1.5. Acid-washed test tubes containing 1.2 ml of the cell suspension were vortexed on a Thermolyne Maxi Mix (Sybron) in the presence of different volumes of hexadecane for 120 s. After phase separation, the absorbance of the lower aqueous phase was measured at 400 nm and compared with that of the cell suspension before the mixing procedure.

General analytical methods. Viscosity was measured in a PSL calibrated Cannon-Ubbelohde Semi-Micro Viscometer (ASTM J.P.; size 50; PSL England). Samples were measured in the standard Tris-magnesium buffer (buffer value, 212 s at 30°C).

For determination of total hexosamine in the medium, cell-free samples (0.1 to 0.5 ml each) were hydrolyzed at 100°C in 5.73 N HCl for 20 min. After drying in vacuo, the samples were dissolved in water and assayed for hexosamine by the indole-hydrochloride method (6). Cell-associated hexosamine was determined on washed cell samples that were suspended in water to 500 to 1,000 Klett units (Klett-Summerson photoelectric colorimeter; green filter). For *A. calcoaceticus* RAG-1, 1,000 Klett units corresponds to 4.8 mg (dry weight)/ml. The samples were hydrolyzed at 100°C in 5.73 N HCl for 20 min, neutralized with NaOH, and assayed for hexosamine by the indole-hydrochloride method (6).

Reducing sugar was determined by the ferricyanide method (25), with D-galactosamine as the standard. Protein was estimated by the method of Lowry et al. (21), with bovine serum albumin as the standard. The ester content of emulsan was determined by a modification (35) of the Hestrin method (13), with acetohydroxymate as the standard. Cell dry weight was determined after drying of a sample of washed cells overnight at 80°C.

RESULTS

Enrichment culture for emulsan-degrading bacteria. After several transfers in an enrichment culture medium containing emulsan as the source of carbon and energy, a mixed bacterial population was obtained that could depolymerize emulsan and use it as a nutrient for growth. The time course of bacterial growth and emulsan degradation by the mixed culture is summarized in Table 1. Growth, measured by increase in culture turbidity and total cell number of the mixed bacterial population, occurred in two distinct steps. During the initial 13 h of incubation, turbidity and cell number increased 12- and 14-fold, respectively. After a lag of at least 11 h, during which time there was no net increase in turbidity or cell number, growth of the culture again took place, leading to another three- to fivefold increase in bacteria by 97 h.

Since the decrease in extracellular amino sugar (a measure of emulsan polysaccharide) became significant only after 24 h, the initial growth phase (0 to 13 h) was probably at the expense of residual protein or other impurities in

TABLE 1. Bacterial growth and emulsan degradation by a mixed culture

Time after inoculation (h)	Growth[a]		Extracellular supernatant fluid[b]		
	A_{560}	Cells/ml	Reduced viscosity (cm^3/g)	Emulsifying activity (%)	Amino sugar (μg/ml)
0	0.01	1.0×10^7	358	100	127
13	0.12	1.4×10^8	245	90	117
24	0.11	1.5×10^8	43	11	125
37	0.25	2.8×10^8	10	15	110
48	0.29	2.8×10^8	4.5	9	63
62	0.34	5.6×10^8	6.4	8	62
97	0.47	4.3×10^8	0	5	33

[a] A mixed bacterial population obtained by enrichment culture on emulsan medium was inoculated into MS buffer containing 0.73 mg of emulsan per ml. Culture turbidity (absorbance at 560 nm [A_{560}]) and total bacteria per milliliter were determined after incubation with shaking at 30°C.

[b] Samples of the mixed culture were centrifuged to remove cells; viscosity, emulsifying activity, and total amino sugar content of the supernatant fluid were determined as described in the text.

the emulsan preparation. During the lag phase (13 to 24 h), both viscosity and emulsifying activity of the extracellular fluid decreased sharply. Only during the second growth phase, beginning at 24 h, was the amino sugar consumed. Since depolymerization preceded hexosamine utilization, low-molecular-weight hexosamine compounds could have served as the carbon source for microorganisms lacking emulsanase activity.

Depolymerization of emulsan by the mixed culture was due, at least in part, to the production of an extracellular emulsan depolymerase(s). As seen in Fig. 1, the addition of cell-free supernatant fluid from the mixed culture brought about a rapid reduction in the viscosity of an emulsan solution. A control containing boiled supernatant fluid did not affect the viscosity of the emulsan solution.

Isolation of a pure culture of an emulsan-degrading bacterium from the mixed culture. From the above results it was clear that the mixed culture contained at least one type of microorganism capable of producing an extracellular emulsanase. To isolate the desired emulsan-degrading bacterium, samples from the mixed culture were spread onto nutrient agar. After incubation at 30°C for 48 h, 20 different colony types were selected, and each was examined for emulsan-degrading ability. Although several of the 20 isolates grew to some extent on emulsan medium, none of them reduced the viscosity of the medium. Furthermore, when the 20 isolates were inoculated together into emulsan medium and incubated for 30°C for 1 week, there was still no drop in the viscosity of the medium.

These negative results prompted an examination of the frequency of emulsan-degrading bacteria in the mixed culture (Table 2). This approach was used to obtain a rough estimate of the number of colonies which would have to be screened to obtain the desired microorganism. The smallest inoculum size still capable of inactivating emulsan, even after 2 weeks of incubation, contained 8×10^2 viable cells per ml. Thus, the frequency of emulsanase-positive bacteria in the mixed culture was likely to be between 0.01 and 0.001.

Since many colonies appeared similar, it was possible that careful screening of 50 different isolates would yield the relevant organism. In fact, in a second larger screening experiment, 1 of 50 isolates examined was able to degrade emulsan, as determined by loss in both emulsifying activity and viscosity of the medium. The emulsan-degrading strain, referred to as YUV-1, is an aerobic, gram-negative, non-spore-forming, non-photosynthetic, rod-shaped bacterium about 4 μm in length and 1 μm in diameter, with rounded to pointed ends. Bacteria usually ap-

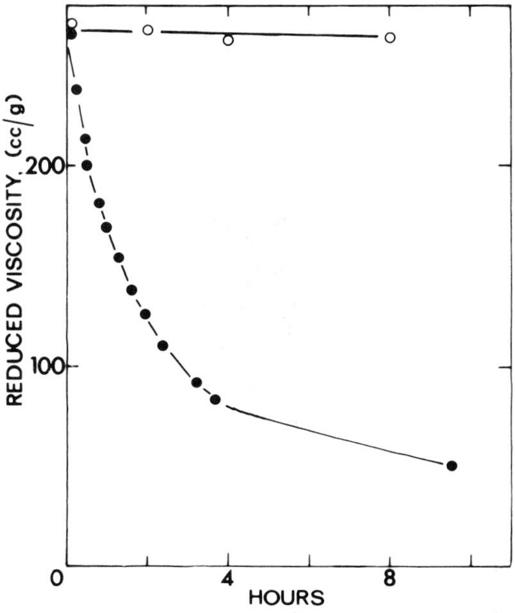

FIG. 1. Depolmerization of emulsan by a cell-free supernatant fluid from the mixed culture. A mixed culture grown on emulsan medium for 40 h was centrifuged, and the supernatant fluid was then filtered through a 0.45-μm filter. This cell-free supernatant fluid was added directly to an equal volume of an emulsan solution (1.46 mg/ml); the viscosity of the mixture (●) and that of the control containing boiled supernatant fluid (○) were determined at various times of incubation at 30°C.

TABLE 2. Emulsan degradation by serial dilutions of the mixed culture

Inoculum[a] (bacteria/ml)	Emulsan degradation[b]			
	15 h	48 h	96 h	2 weeks
8×10^7	+	+	+	+
8×10^6	+	+	+	+
8×10^5	−	+	+	+
8×10^4	−	+	+	+
8×10^3	−	−	+	+
8×10^2	−	−	+	+
8×10^1	−	−	−	−
8×10^0	−	−	−	−

[a] The mixed culture grown on emulsan medium for 3 days was serially diluted into 1 ml of fresh emulsan medium. Incubation was in small test tubes with shaking at 30°C. The initial cell concentration was determined by plating on LB agar.

[b] Emulsan degradation was measured by the qualitative micromethod. +, Emulsan was degraded; −, emulsan was not degraded.

pear as singles, although some occur in short chains, up to three in length. Exponentially growing cells in LB medium were actively motile. Strain YUV-1 produced small colonies (1 to 2 mm in diameter) on LB agar after 2 days at 32°C; the glistening colorless colonies were raised and circular with smooth edges. The cells were catalase positive and nonhemolytic; gelatin and starch were not hydrolyzed. Growth did not occur on azide plates; growth on MacConkey plates was poor. The optimum growth temperature was between 32 and 40°C. Strain YUV-1 grew with a doubling time of about 2.5 h in medium containing 0.5 to 1.5% yeast extract but failed to grow in nutrient broth (Difco) or minimal medium containing glucose or acetate as the sole carbon source. When 0.5% glucose was added to yeast extract medium, there was a stimulation in growth, decrease in pH, and a characteristic formation of flocs of cells encased in an organic matrix. The above properties, especially the formation of flocs at high C to N ratios in the growth media, suggest that strain YUV-1 is a member of the genus *Zoogloea* (8).

Growth of YUV-1 on lawns of *A. calcoaceticus* RAG-1 and degradation of cell-bound emulsan. *A. calcoaceticus* RAG-1 forms dull white colonies on ethanol agar, whereas mutants of RAG-1 incapable of producing emulsan form white translucent colonies (26). Strain YUV-1, when placed on preformed lawns of RAG-1, produced translucent plaques (Fig. 2). The plaques appeared after 24 h at 30°C and continued to grow until the entire plate was covered. The plaques increased in diameter about 2.5 mm per 12 h. RAG-1 cells within the plaque appeared to have the same translucent phenotype as the emulsan-negative mutants. When RAG-1 was taken from within the plaque and placed on fresh ethanol agar, the cells grew and regained their original wild-type appearance. Two to three days after inoculation of RAG-1 lawns with YUV-1, it was possible to observe a thin layer of YUV-1 cells in the center of each of the plaques (Fig. 2). Plaque formation appeared only on emulsan-containing RAG-1 cells. Emulsan-negative mutants AG-1, TR3, and 10, as well as the rhamnose-glucose encapsulated *A. calocoaceticus* BD4, did not allow plaque formation. An example of strain specificity for plaque formation is shown in Fig. 3.

Direct evidence that YUV-1 solubilized the emulsan capsule from RAG-1 cells, thereby forming turbid plaques, was obtained by determining cell-associated hexosamine before and after treatment of RAG-1 with YUV-1 (Table 3). RAG-1 cells within the plaque contained only 17.5 μg of hexosamine per mg of cells (dry weight), whereas the untreated control contained 53.3 μg of hexosamine per mg of cells (dry weight). If the hexosamine content of the emulsan-negative mutant AG-1 (presumably due to cell wall material) is subtracted from the treated and untreated RAG-1 values, the percent reduction of emulsan was [(43.4 − 7.6)/(43.4)] × 100 = 82%.

As a consequence of the solubilization of the emulsan capsule by YUV-1, the RAG-1 cells became more hydrophobic. YUV-1-treated RAG-1 cells adhered with greater affinity to hexadecane than untreated cells over the entire

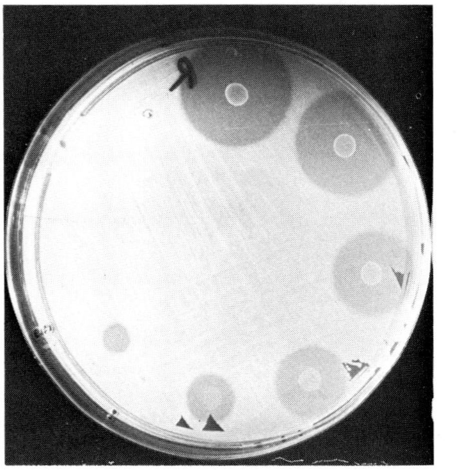

FIG. 2. Kinetics of plaque formation by strain YUV-1 on lawns of *A. calcoaceticus* RAG-1. A preformed lawn of RAG-1 cells was prepared by spreading RAG-1 with a swab on ethanol agar and incubating for 12 h at 32°C. A fixed inoculum (10 μl of 6×10^7 YUV-1 per ml) was introduced every 12 h, starting at the top of the plate and moving clockwise. The photograph was taken 108 h after the initial inoculation.

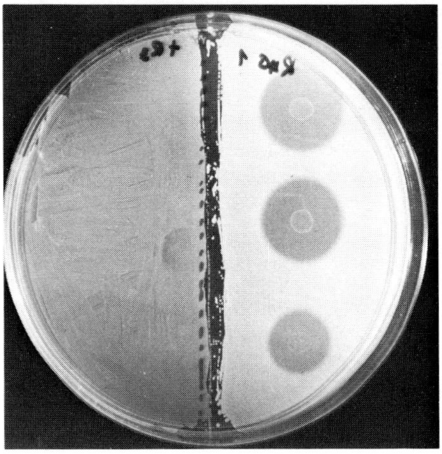

FIG. 3. Strain specificity for plaque formation by strain YUV-1. Preformed lawns of *A. calcoaceticus* RAG-1 (right) and TR3 (left) were prepared on an ethanol agar plate. Each side of the plate was then inoculated with 6×10^5 (upper), 6×10^4 (middle), and 6×10^3 (bottom) YUV-1 cells. The photograph was taken after 60 h. Note the translucent appearance of the TR3 lawn.

range of hexadecane volumes tested (Fig. 4). With 0.2 ml of the hydrocarbon, 93% of the treated cells were adherent, whereas only 61% of the control RAG-1 cells were bound to the hexadecane-water interface.

Degradation of emulsan by YUV-1. Growth of a pure culture of YUV-1 on a medium containing yeast extract and emulsan is shown in Fig. 5. Growth, measured either by culture turbidity or viable cell count (data not shown), occurred in two distinct phases. During the first day, cell concentration increased about 10-fold. This initial growth phase must have been at the expense of certain components in the yeast extract because there was no evidence of any emulsan degradation. During the lag in growth (24 to 48 h), extensive depolymerization of emulsan occurred, although total extracellular hexosamine did not decrease. It follows that low-molecular-weight emulsan degradation products accumulated during this period. During the second growth phase (48 to 70 h), hexosamine was consumed.

TABLE 3. Cell-associated emulsan content of YUV-1-treated RAG-1 cells[a]

A. calcoaceticus strain	μg of hexosamine per mg of cells (dry wt)	Emulsan[b] (%)
RAG-1	53.3	17.4
AG-1 (emulsan negative)	9.9	0
YUV-1-treated RAG-1	17.5	3.0

[a] *A. calcoaceticus* RAG-1 and AG-1 were spread onto ethanol agar plates and incubated at 30°C. After 24 h, some of the plates containing lawns of RAG-1 were inoculated with YUV-1 as described in the text. All of the plates were then incubated for an additional 3 days at 30°C. Bacteria were then harvested from the plates, washed twice with cold water, and then suspended in water to ca. 1,000 Klett units for determinations of cell dry weight and hexosamine content.

[b] The percentage of cell dry weight that is emulsan was calculated by assuming that (i) 9.9 μg of hexosamine per mg of cells (dry weight) is due to cell wall material (analysis of the emulsan-negative mutant AG-1), and (ii) acid-hydrolyzed pure emulsan yields 25% hexosamine by this test (32).

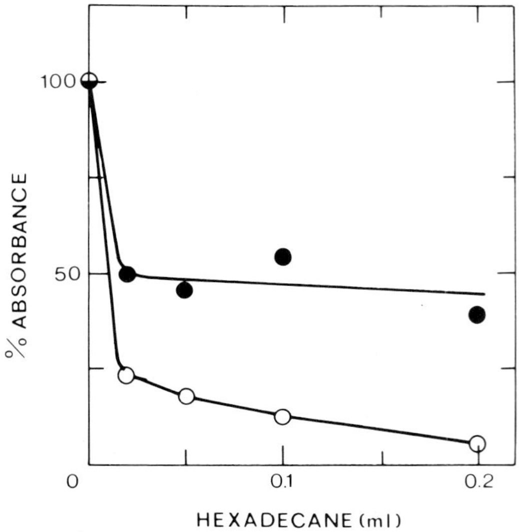

FIG. 4. Adherence of YUV-1-treated *A. calcoaceticus* RAG-1 cells to hexadecane. RAG-1 cells from ethanol agar plates were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4 to 1.5. Bacterial suspensions (1.2 ml) were vortexed in the presence of different volumes of hexadecane. After phase separation, the turbidity of the lower aqueous phase was measured. Results are expressed as percentage of the initial absorbance of the lower aqueous phase after the mixing procedure as a function of the hexadecane volume added. Symbols: ●, RAG-1; ○, YUV-1-treated RAG-1.

DISCUSSION

Starting with the pioneer studies of Dubos and Avery (7), a number of investigators have isolated different soil bacteria capable of hydrolyzing capsular polysaccharides (2, 12, 18, 22, 23, 34, 38). Although a mixed population of microorganisms capable of degrading emulsan was readily obtained by standard enrichment culture procedures, difficulties were encountered in isolating the particular microorganisms which produced the degrading enzyme. Only after extensive screening was the emulsan-degrading bacterium, strain YUV-1, isolated from the mixed culture.

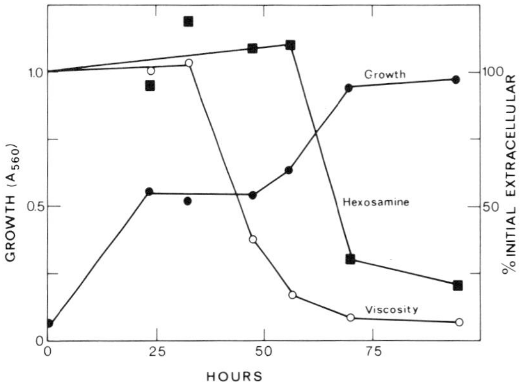

FIG. 5. Growth of YUV-1 on emulsan-yeast extract medium. One milliliter of an overnight culture of YUV-1 in LB medium was inoculated into 30 ml of MS buffer containing 0.8 mg of emulsan per ml and 5 mg of yeast extract per ml. After incubation at 30°C with shaking, 3-ml samples were removed for determination of culture turbidity (absorbance at 560 nm [A_{560}]) and then centrifuged to remove cells. Viscosity and total amino sugar content of the supernatant fluid were determined as described in the text.

Studying YUV-1 in pure culture made possible an understanding of some of the difficulties encountered in the isolation procedure. In addition to the fact that YUV-1 was a minor constituent (<1%) in the mixed population, the strain grew very slowly on nutrient agar, forming small colonies only after 4 to 5 days. An even greater difficulty arose from the fact that pure cultures of YUV-1 do not grow at a significant rate on emulsan media. Although YUV-1 can utilize emulsan, glucose, and acetate as carbon and energy sources, the strain requires additional factors (present in yeast extract) for growth. It is likely that other bacteria in the mixed culture provided these growth factors for YUV-1 in the initial enrichment cultures. Synergistic relationships involving bacteria are well known (24) and include hemicellulose utilization (4), growth on methane (33), and complementation of lecithinase activity by two *Pseudomonas* strains (1).

Strain YUV-1 produced growing translucent plaques only when spotted on lawns of emulsan-encapsulated bacteria, suggesting that plaque formation was due to an extracellular, diffusible emulsanase. The RAG-1 cells inside the plaque were shown by chemical analysis to be decapsulated, although they remained viable and were able to regain their initial colonial morphology when transferred to fresh media. This phenomenon is similar in appearance to the well-known case of phage-induced capsule-degrading enzymes producing halos on lawns of encapsulated bacteria (20).

Previously, it was shown that RAG-1 cells grown under conditions that lead to the accumulation of emulsan on the cell surface adhere poorly to hydrocarbons (27a). In addition, the emulsan-deficient mutant TR3 adhered better to hexadecane than did the parent RAG-1 cells. These data led to the hypothesis that the emulsan capsule interfered with adherence to hydrocarbons. The fact that enzymatic decapsulation of RAG-1 increased its cell surface hydrophobicity (Fig. 4) provides additional evidence for this concept. Adherence of RAG-1 cells is due, at least in part, to the presence of thin fimbriae (30).

Although the isolation and characterization of the enzymes responsible for emulsan degradation will be the subject of a separate report, certain properties of these enzymes can be derived from the growth experiments reported here. Emulsanase activity was produced by YUV-1 growing (i) on lawns of emulsan-encapsulated RAG-1 cells and (ii) in yeast extract-emulsan medium. However, emulsanase activity was not present (as determined by plaque formation or decrease in viscosity of emulsan solutions) when YUV-1 was grown on yeast extract alone, yeast extract plus glucose, or LB medium. Thus, emulsanase production appears to be induced by emulsan. At least part of the degradative activity must be extracellular because (i) the enzyme is diffusible in agar, and (ii) cell-free supernatant fluids from YUV-1 cultures brought about a rapid drop in the viscosity of emulsan solutions.

The demonstration that emulsan can be degraded by soil bacteria indicates that emulsan will be effective in an open system for only a limited time period. Isolation of emulsanase from YUV-1 should provide a valuable tool for (i) further studies on the chemical structure of emulsan and (ii) specifically breaking hydrocarbon-in-water emulsions stabilized by emulsan.

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LITERATURE CITED

1. **Bates, J. L., and P. V. Liu.** 1963. Complementation of lecithinase activities by closely related pseudomonads and its taxonomic implication. J. Bacteriol. **86:**385–592.
2. **Becker, G., and A. M. Pappenheimer.** 1966. Lyase activity of inducible S8-depolymerases from *Bacillus palustris*. Biochim. Biophys. Acta **121:**343–348.
3. **Belsky, I., D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. **101:**175–178.
4. **Coen, A. J., and A. B. Dehority.** 1970. Degradation and utilization of hemicellulose from intact forages by pure cultures of rumen bacteria. Appl. Microbiol. **20:**362–368.
5. **Cooper, D. G., and J. E. Zajic.** 1980. Surface-active compounds from microorganisms. Adv. Appl. Microbiol. **26:**229–253.
6. **Dische, Z.** 1955. New color reactions for determination of

sugars in polysaccharides. Methods Biochem. Anal. **2**:313–358.

7. **Dubos, R. J., and O. Avery.** 1931. Decomposition of the capsular polysaccharide of pneumococcus type III by a bacterial enzyme. J. Exp. Med. **54**:51–71.
8. **Dugan, P. R.** 1981. The genus *Zoogloea*, p. 764–770. *In* M. P. Starr, H. Stolop, H. G. Truper, A. Balawr, and H. G. Schlegel (ed.), The procaryotes. Springer-Verlag, Berlin.
9. **Erickson, I. E., and T. Nakahara.** 1975. Growth in cultures with two liquid phases: hydrocarbon uptake and transport. Proc. Biochem. **10**:9–13.
10. **Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg, and D. L. Gutnick.** 1982. Emulsan in *Acinetobacter calcoaceticus* RAG-1: distribution of cell-free and cell-associated cross reacting material. Appl. Environ. Microbiol. **44**:165–170.
11. **Gutnick, D. L., and E. Rosenberg.** 1977. Oil tankers and pollution: a microbiological approach. Annu. Rev. Microbiol. **31**:379–396.
12. **Harada, T., K. Moori, and A. Amemura.** 1972. Highly specific, inducible β-glucanases from *Flavobacterium*. Agric. Biol. Chem. **36**:2611–2613.
13. **Hestrin, S.** 1949. Reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. **180**:249–261.
14. **Hisatsuka, K., T. Nakahara, N. Sono, and K. Yamada.** 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem. **35**:686–692.
15. **Iguchi, T., I. Takeda, and H. Ohsawa.** 1969. Emulsifying factor of hydrocarbon produced by a hydrocarbon assimilating yeast. Agric. Biol. Chem. **33**:1657–1658.
16. **Itoh, S., H. Honda, F. Tomita, and T. Suzuki.** 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* growth on n-paraffin. J. Antibiot. **24**:855–859.
17. **Juni, E., and A. Janick.** 1969. Transformation of *Acinetobacter calcoaceticus* (*Bacterium antitratum*). J. Bacteriol. **98**:281–288.
18. **Kaplan, N., and E. Rosenberg.** 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. Appl. Environ. Microbiol. **44**:1335–1341.
19. **Lesley, S. M.** 1961. Degradation of the polysaccharide of *Xanthomonas phaseoli* by an extracellular bacterial enzyme. Can. J. Microbiol. **7**:815–825.
20. **Lindberg, A. A.** 1977. Bacterial surface carbohydrates and bacteriophage adsorption, p. 289–356. *In* I. W. Sutherland (ed.), Surface carbohydrate of the prokaryotic cell. Academic Press, Inc., London.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Manners, D. J.** 1955. The enzymatic degradation of polysaccharides. Quant. Rev. **9**:73–99.
23. **McNicol, L. A., and E. A. Baker.** 1970. Pectate lyase and polygalacturonic lyase activity in V_i antigen-degrading enzyme preparation. Biochemistry **9**:1017–1023.
24. **Meers, L. J.** 1973. Growth of bacteria in mixed cultures. Crit. Rev. Microbiol. **2**:139–184.
25. **Park, J. T., and M. J. Johnson.** 1949. A submicro-determination of glucose. J. Biol. Chem. **181**:149–151.
26. **Pines, O., and D. L. Gutnick.** 1981. Relationship between phage resistance and emulsan production, interaction of phages with the cell-surface of *Acinetobacter calcoaceticus*. Arch. Microbiol. **130**:129–133.
27. **Reisfeld, A., E. Rosenberg, and D. L. Gutnick.** 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. **24**:363–368.

27a. **Rosenberg, E., N. Kaplan, O. Pines, M. Rosenberg, and D. Gutnick.** 1983. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbon. FEMS Microbiol. Lett. **17**:157–160.

28. **Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. **37**:404–413.
29. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
30. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. Appl. Environ. Microbiol. **44**:929–937.
31. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.
32. **Rubinovitz, C., D. L. Gutnick, and E. Rosenberg.** 1982. Emulsan production by *Acinetobacter calcoaceticus* RAG-92 in the presence of chloramphenicol. J. Bacteriol. **152**:126–132.
33. **Sheenan, B. T., and M. J. Johnson.** 1971. Production of bacterial cells from methane. Appl. Microbiol. **21**:511–515.
34. **Sickles, G., and M. Shaw.** 1935. A microorganism which decomposes the specific carbohydrate of pneumococcus type VIII. Proc. Soc. Exp. Biol. Med. **32**:857–858.
35. **Stern, J., and B. Shapiro.** 1953. A rapid and simple method for the determination of esterified fatty acids and for total fatty acids in blood. J. Clin. Pathol. **6**:158–160.
36. **Sutherland, I. W.** 1979. Microbial exopolysaccharides: control of synthesis and acylation, p. 1–34. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), Microbial polysaccharides and polysacchardses. Academic Press, Inc., London.
37. **Taylor, W. H., and E. Juni.** 1961. Pathways for biosynthesis of a bacterial polysaccharide. I. Characterization of the organism and polysaccharide. J. Bacteriol. **81**:688–693.
38. **Torriani, A. M., and A. M. Pappenheimer.** 1962. Inducible polysaccharide depolymerases of *Bacillus palustris*. J. Biol. Chem. **237**:3–13.
39. **Zajic, J. E., H. Guignard, and D. F. Gerson.** 1977. Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus*. Biotech. Bioeng. **19**:1285–1301.
40. **Zajic, J. E., and C. J. Panchal.** 1976. Bioemulsifiers. Crit. Rev. Microbiol. **5**:39–66.
41. **Zosim, Z., D. Gutnick, and E. Rosenberg.** 1981. Properties of hydrocarbon-in-water emulsions stabilized by emulsan. Biotech. Bioeng. **24**:281–292.
42. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. **37**:414–420.

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Hypothesis

Bacterial adherence to hydrocarbons and to surfaces in the oral cavity

Mel Rosenberg [+], Eugene Rosenberg [*], Herbert Judes and Ervin Weiss

School of Dental Medicine and Department of Human Microbiology [+], Sackler Faculty of Medicine, and [*] Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Israel 69978

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1. INTRODUCTION

At first glance, the adherence of bacteria to oil droplets and bacterial attachment to surfaces within the mouth appear to have little in common. Until recently, investigators proposed that adherence to hydrocarbons is a property limited to paraffin-oxidizing bacteria and yeast [1–3]. Similarly, most recent investigations into the attachment of bacteria within the oral cavity have postulated stereospecific, molecular recognition mechanisms as the major factor involved [4]. However, experiments conducted in several laboratories during the past few years have suggested an alternate view to both these phenomena: that both bacterial adherence to hydrocarbons, and to oral surfaces, depend primarily on hydrophobic interactions. This hypothesis is discussed in the present report.

2. BACTERIAL ADHERENCE TO HYDROCARBONS

Bacterial partition at the oil : water interface was reported almost 60 years ago, in the classic investigations of Mudd and Mudd [5,6]. These authors first suggested that the hydrophobic surface properties of bacteria were responsible for this phenomenon. The oriented partitioning of *Flexibacter, Hyphomicrobium* and *Rhizobium* sp. at the aqueous : oil interface has been reported by Marshall and coworkers [7,8]. Yet despite these observations, microbiologists studying growth of bacteria and yeast on oil have often suggested that adherence to hydrocarbon is a unique property of petroleum degrading microorganisms, and dependent on some specific recognition mechanism between the cell and its insoluble substrate [1–3]. The development of simple techniques for measuring bacterial adherence to hydrocarbons (BATH) [9,10] and isolating nonadherent mutants [11] enabled us to address this question in a quantitative manner. It soon became apparent that adherence to liquid hydrocarbons was not limited to oil-degrading bacteria [9]. Moreover, several oil-degrading strains, e.g., *Pseudomonas aeruginosa*, exhibited low affinity towards hydrocarbons, even following growth on hexadecane [10]. In addition, *Acinetobacter calcoaceticus* RAG-1, an oil-degrading marine bacterium [12], adhered avidly not only to hexadecane, which it is capable of metabolizing, but also to other liquid hydrocarbons which it cannot degrade; i.e., octane and xylene [9]. Although experiments were later carried out which underlined the importance of adherence in growth of RAG-1 on hydrocarbon [11], the possibility of a substrate-specific adherence mechanism was no longer tenable. These results led us to postulate that BATH is primarily a measure of cell surface hydrophobicity and can be employed as a useful

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general tool for studying bacterial hydrophobicity [9].

Several investigations have shown that BATH correlates well with other techniques for measuring bacterial hydrophobicity, including adherence to hydrophobic Sepharose beads [13] and to polystyrene [14]. Similarly, reports of cell-surface hydrophobicity of various bacteria, e.g., *Serratia* [9,15], staphylococci [9,16,17], *Streptococcus pyogenes* [18,19] and bacillus spores [20,21], rough enterobacterial strains [22], e.g., *Proteus mirabilis* [23], *Salmonella typhimurium* and *Escherichia coli* [9], have been corroborated by BATH. These data have contributed to the growing amount of information implicating hydrophobic interactions in various adherence phenomena, both in the environment and in the host. Perhaps the most intriguing of these is the possible importance of bacterial hydrophobicity in mediating attachment within the oral cavity. This is discussed in the following section.

3. BACTERIAL ADHERENCE IN THE ORAL CAVITY

The axiom that bacteria must adhere in order to prevent removal from their niche is perhaps nowhere as evident as in the mouth. Whereas the mouth represents a hospitable bacterial growth chamber offering a wide variety of microniches, dislodgement results in the removal of bacteria to inhospitable surroundings; i.e., the stomach. Very high numbers of bacteria may be found adhering to all oral surfaces, but in particular to the non-desquamating tooth surface [4].

Studies on the possible role of hydrophobic interactions in mediating adherence within the oral cavity have thus far concentrated on two major surfaces: human buccal epithelial cells and the tooth surface.

3.1. Adherence to buccal epithelial cells

In an initial study on the role of bacterial hydrophobicity in adherence to human buccal epithelial cells, adherence of the non-oral strain, *A. calcoaceticus* RAG-1 was studied [19]. Adherence of RAG-1 to buccal epithelial cells was directly related to their ability to adhere to hydrocarbon. Moreover, an isogenic mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was similarly unable to adhere to the epithelial cells. *Streptococcus pyogenes* M-5, a pathogen and colonizer of the oral mucosa, was shown to adhere avidly to hexadecane. More recently, the effect of the amphipathic polysaccharide, emulsan, on bacterial adherence to human buccal epithelial cells was studied [24]. Emulsan forms a thin polymeric film on hydrocarbon droplets [25], thereby preventing and reversing adherence of hydrophobic bacteria; e.g., *A. calcoaceticus* RAG-1 and *S. pyogenes* M-5. The fact that emulsan was similarly able to inhibit attachment of these strains to epithelial cells provided further evidence for the importance of hydrophobic interactions in mediating their adherence to epithelial cells. Of particular interest was the finding that the desorption ability of emulsan extends to the indigenous normal flora on the buccal epithelial cells: emulsan was able to desorb over 70% of the normal flora from over 500 epithelial cells scored [24].

3.2. Adherence to the human tooth surface

Colonization of the tooth surface begins with an initial population of bacteria which adhere directly to the tooth surface, subsequently leading to the formation of a macroscopic, multilayered film of attached bacteria known as supragingival dental plaque [4,26]. In order to obtain an estimate of the proportion of hydrophobic bacteria on the tooth surface, we initially chose an approach similar to that used by Dahlback and coworkers to study the adherent properties of bacteria at the air : water interface [27]. Fresh isolates of bacteria were obtained by removing adherent cells from washed, extracted teeth by mild sonic oscillation. Among the 112 bacterial isolates tested, 80% adhered to hexadecane [28]. Hydrophobic isolates included streptococci, filaments, rods and coryneforms. This study demonstrated that a variety of hydrophobic bacteria are obtainable in high proportions from the tooth surface. In order to overcome possible criticism that the high percentage of hydrophobic isolates in the study resulted from (i) disproportionate sampling [26], or (ii) cultivation of cells under physiological conditions different from those in situ, BATH was performed using dispersed plaque sample obtained directly from the mouths of volunteers [28]. Adherence of the heterogeneous plaque population was followed turbidimetrically, and by direct microscopic observation. Results demonstrated that the majority of plaque microorganisms exhibit pronounced hydrophobic cell-surface properties in situ.

In order to determine whether hydrophobic interactions can mediate attachment of a non-oral species to the tooth surface, adherence of RAG-1 was compared with that of its isogenic nonhydrophobic mutant, MR-481. RAG-1 cells adhered avidly to the human tooth surface, both in vitro and in situ, whereas MR-481 cells were nonadherent (E. Weiss, H. Judes and M. Rosenberg, in preparation).

Investigators in several laboratories have tested oral laboratory strains for adherence to hexadecane [13,30–35]. In all cases a high proportion of hydrophobic strains was found. Correlations have also been demonstrated between BATH and adherence to saliva-coated hydroxyapatite [31,32, 34,35].

4. THE ROLE OF HYDROPHOBIC INTERACTIONS IN MEDIATING BACTERIAL ADHERENCE WITHIN THE ORAL CAVITY

On the basis of the above-stated data, we submit the hypothesis that hydrophobic interactions are primarily responsible for mediating the adherence of most oral bacteria to the various surfaces within the oral cavity. Several corollaries and predictions follow from this premise:

(A) It has previously been postulated that specific lectin-like interactions are responsible for adherence of bacteria to salivary pellicles [4]. In our opinion, most oral strains can adhere by virtue of their cell-surface hydrophobicity to the various saliva-bathed tissues in the oral cavity, without requiring specific receptor recognition. Colonization would thus largely depend on the bacterial growth conditions prevailing on the various surfaces, rather than on a heterogeneity of adherence receptors. The ability of RAG-1 cells (but not MR-481) to adhere avidly to epithelial cells and to teeth suggests that hydrophobic interactions per se can mediate adherence in the absence of stereospecific recognition mechanisms. The observation that emulsan desorbs the majority of bacteria attached to buccal epithelial cells [24] provides further evidence in support of this view. It may be that lectin-like interactions act primarily to promote bacteria–bacteria interactions within the oral cavity [31].

(B) Certain amphipathic molecules and other agents which disrupt hydrophobic interactions may prove effective in desorbing bacteria from the tooth surface in particular and the oral cavity in general. It is of interest to note in this context that most dentifrices contain a high percentage of detergent (about 2%). It has been claimed that detergents in dentifrices have no biological or prophylactic role, but rather serve a psychological effect as foaming agents [36]. In the light of the above, such a premise must be reconsidered.

(C) Non-toxic oils and hydrocarbons may be able to remove plaque microorganisms from the tooth surface by preferential adsorption by the hydrophobic liquid phase. Hydrocarbons and oils are able to desorb bacteria bound to polystyrene with high efficiency [37].

(D) Recent studies have shown that various suspected oral pathogens (e.g., *Streptococcus sanguis, S. mutans, S. pyogenes* and *Actinomyces viscosus*) exhibit pronounced cell-surface hydrophobicity [3,18,19,30–35]. These data, taken together with our own results on the overall hydrophobicity of plaque microorganisms, suggest that relatively 'benign' oral flora adhere to surfaces in the oral cavity in much the same manner which enables the adherence of pathogenic or cariogenic strains. Thus, agents intended for the removal of deleterious oral bacteria (e.g., detergents) may also desorb high proportions of the normal hydrophobic oral flora.

(E) Since hydrophobic interactions are influenced by the nature of the aqueous medium, as well as the attachment substratum, various properties of saliva, such as pH, ionic strength, and presence of surface-active agents, may act to promote or inhibit adherence of hydrophobic bacteria to surfaces within the oral cavity.

5. SUMMARY

The possibility that hydrophobic interactions play a major role in mediating bacterial attachment within the oral cavity is not surprising, when considered in a larger perspective. Various lines of investigation have implicated bacterial hydrophobicity in a wide variety of adherence phenomena, including phagocytosis [38], adherence at the air : water interface [20,27], adherence to marine sediment [39], adherence to intestinal mucosal cells [22], adherence and growth on hydrocarbons [11], adherence to non-wettable plastics [14], and attachment to plant root hairs [8]. Surfaces in contact with water tend to adsorb a layer of organic molecules which renders the interface with low energy characteristics; the oral cavity is no exception [40].

Bacterial attachment to oil and within the mouth may reflect a general microbial phenomenon: hydrophobic surface components enable bacteria to escape from the bulk aqueous phase to interfaces which offer nutrients and protection.

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REFERENCES

[1] Gutnick, D.L. and Rosenberg, E. (1977) Annu. Rev. Microbiol. 31, 379–380.

[2] Miura, Y., Okazaki, M., Hamada, S.-I., Murakawa, S.-I. and Yugen, R. (1977) Biotechnol. Bioeng. 19, 701–714.

[3] Kennedy, R.S., Finnerty, W.R., Sudarsanan, K. and Young, R.A. (1975) Arch. Microbiol. 102, 75–83.

[4] Gibbons, R.J. (1980) in Microbial Adhesion to Surfaces (Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P. and Vincent, B., Eds.) pp. 351–388, Ellis Horwood, Chichester.

[5] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 633–645.

[6] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 647–660.

[7] Marshall, K.C. and Cruickshank, R.H. (1973) Arch. Mikrobiol. 91, 29–40.

[8] Marshall, K.C., Cruickshank, R.H. and Bushby, H.V.A. (1975) J. Gen. Microbiol. 91, 198–200.

[9] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[10] Rosenberg, M., Rosenberg, E. and Gutnick, D. (1980) in Microbial Adhesion to Surfaces (Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P. and Vincent, B., Eds.) pp. 541–542, Ellis Horwood, Chichester.

[11] Rosenberg, M. and Rosenberg, E. (1981) J. Bacteriol. 148, 51–57.

[12] Reisfeld, A., Rosenberg, E. and Gutnick, D. (1972) Appl. Microbiol. 24, 363–368.

13 Olsson, J. and Westergren, G. (1982) FEMS Microbiol. Lett. 15, 319–323.

[14] Rosenberg, M. (1981) Appl. Environ. Microbiol. 42, 375–377.

[15] Kjelleberg, S., Lagercrantz, C. and Larsson, Th. (1980) FEMS Microbiol. Lett. 7, 225–228.

[16] Hogt, A.H., Dankert, J., Feigen, J., and de Vries, J.A. (1981) Antonie van Leeuwenhoek 48, 496–498.

[17] Jonsson, P. and Wadstrom, T. (1983) Current Microbiol., in press.

[18] Tylewska, S.K., Hjertén, S. and Wadström, T. (1979) FEMS Microbiol. Lett. 6, 249–253.

[19] Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) Infect. Immun. 33, 29–33.

[20] Boyles, W.A. and Lincoln, R.E. (1958) Appl. Microbiol. 6, 327–334.

[21] Rosenberg, M. (1982) Ph.D. thesis, Tel-Aviv University, Ramat-Aviv, Israel.

[22] Edebo, L., Kihlström, E., Magnusson, K.-E. and Stendahl, O. (1980) in Cell Adhesion and Motility (Curtis, A.S.G. and Pitts, J.D., Eds.) pp. 65–103, Cambridge University Press, New York.

[23] Rosenberg, M., Rottem, S. and Rosenberg, E. (1982) FEMS Microbiol. Lett. 13, 167–169.

[24] Rosenberg, E., Gottlieb, A. and Rosenberg, M. (1983) Infect. Immun. 39, 1024–1028.

[25] Zosim, Z., Gutnick, D. and Rosenberg, E. (1982) Biotechnol. Bioeng. 24, 281–292.

[26] Nolte, W.A. (1982) in Oral Microbiology (Nolte, W.A., Ed.) pp. 193–228, C.V. Mosby, St. Louis.

[27] Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. (1981) Arch. Microbiol. 128, 267–270.

[28] Weiss, E., Rosenberg, M., Judes, H. and Rosenberg, E. (1982) Current Microbiol. 7, 125–128.

[29] Rosenberg, M., Judes, H. and Weiss, E., submitted.

[30] Doyle, R.J., Nesbitt, W.E. and Taylor, G. (1982) FEMS Microbiol. Lett. 15, 1–5.

[31] Gibbons, R.J. and Etherden, I., submitted.

[32] Gibbons, R.J., Etherden, I. and Skobe, Z., submitted.

[33] Ofek, I., Whitnack, E. and Beachey, E.H. (1983) J. Bacteriol. 154, 139–145.

[34] Nesbitt, W.E., Doyle, R.J. and Taylor, K.G. (1982) Infect. Immun. 38, 637–644.

[35] Westergren, G. and Olsson, J. (1983) Infect. Immun., in press.

[36] Allen, A.L., Hawley, C.E., Cutright, D.E. and Seibert, J.S. (1975) J. Periodontol. 46, 102–112.

[37] Rosenberg, M., Judes, H. and Weiss, E., submitted.

[38] van Oss, C.J. (1978) Annu. Rev. Microbiol. 32, 19–40.

[39] Shilo, M. and Fattom, A. (1982) Symposium for General Microbiology, Dundee, Sept. 7–9, 1982.

[40] Glantz, P.-O. (1977) Odontologisk Revy 20 (Supplement 17), 1–132.

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Microbial Contamination by a Medical Carbon Dioxide Laser

Ilana Eli, DMD, Herbert Judes, BDS (Rand), and Mel Rosenberg, PhD

Department of Operative Dentistry, School of Dental Medicine (I.E., H.J., M.R.), and Department of Human Microbiology, Sackler Faculty of Medicine (M.R.), Tel-Aviv University, Ramat-Aviv, Israel

One of the advantages in using the carbon dioxide laser in medicine is the sterilization of the wound at the site of surgical intervention. In microbial studies, using the Sharplan Model 743 Medical Laser, we found substantial contamination of the area directly below the probe by viable bacteria and fungi. The levels of contamination varied from experiment to experiment, but were always substantial. The contamination is likely due to the stream of nitrogen gas emitted during and following laser irradiation in order to cool the lens. Following the implementation of several simple prophylactic procedures, including insertion of a filter on the end of the tube emitting the nitrogen gas, contamination by the gas stream was reduced to insignificant levels.

Key words: carbon dioxide laser, microbial contamination, nitrogen gas

INTRODUCTION

The CO_2 laser has created a revolution in various fields of modern medicine. Its application in soft-tissue surgery is widely accepted in many areas, including plastic surgery, oncology, and eye surgery [1–9]. The potential use of the CO_2 laser in the field of hard-tissue intervention (eg, dentistry) is currently under investigation [10–12].

One of the advantages claimed for use of the laser beam as a surgical tool is its sterilization ability [13,14]. The high energy irradiated by the laser beam kills the microbial population at the site of intervention [15], thus lowering the risk of microbial contamination and ensuing infections, enabling many surgical treatments to be carried out as ambulatory procedures.

In spite of the above, preliminary experiments which were conducted in this laboratory dealing with the effects of a medical laser on bacterial populations dem-

Address reprint requests to Dr Ilana Eli, Department of Operative Medicine, School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978 Tel-Aviv, Israel.

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onstrated that the laser contaminates the working surface in the immediate area of the irradiating probe. The source of the contamination was found to be the stream of gas which is emitted during irradiation in the vicinity of the laser lens, in order to cool the lens. In this report, this potentially harmful phenomenon is described, as well as the simple measures taken to overcome it.

MATERIALS AND METHODS

The Sharplan Model 743 Medical Laser, used in these experiments, is shown in Figure 1. This carbon dioxide laser is capable of high-energy outputs (up to 80 W), and both continuous and single-beam emissions ranging from 0.01 to 0.5 seconds in duration. One of the general technical problems related to use of carbon dioxide lasers at high energies involves overheating of the lens. This may be controlled by various means. In the Sharplan laser, nitrogen gas is streamed in the vicinity of the probe, during and immediately following irradiation, for a total duration of 3.6 seconds. The stream of gas is emitted through the hand-held probe in the direction of the working surface.

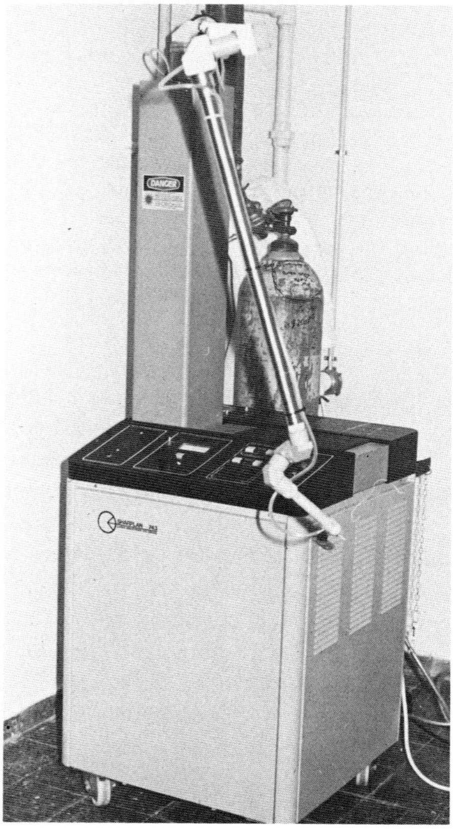

Fig. 1. Carbon dioxide Sharplan Model 743 Medical Laser used in these experiments.

In our bacteriological experiments with the Sharplan laser, contamination problems were routinely encountered. In order to further examine this problem, sterile agar plates containing bacteriological growth medium (brain heart infusion agar, Difco Ltd) were placed beneath the laser probe, at a distance of 6 cm from focus. Plates were opened and exposed to 60 or 120 seconds of gas emission, with the laser energy setting on 0 W. Plates were incubated for 48 hours at 30°C under aerobic conditions and scored for microbial colonies. Contamination was expressed as the number of colony-forming units per 120 seconds of gas emission. As controls, identical sterile agar plates were opened to the atmosphere in the same room for equal durations of time. Contamination of the control plates was less than 2% of that observed for the plates exposed to gas emission through the laser probe.

RESULTS AND DISCUSSION

Typical results obtained from exposure of the agar plates to the stream of gas emitted from the laser probe are shown in Figures 2 and 3. Whereas a large variation was observed in experiments performed on different dates, substantial contamination

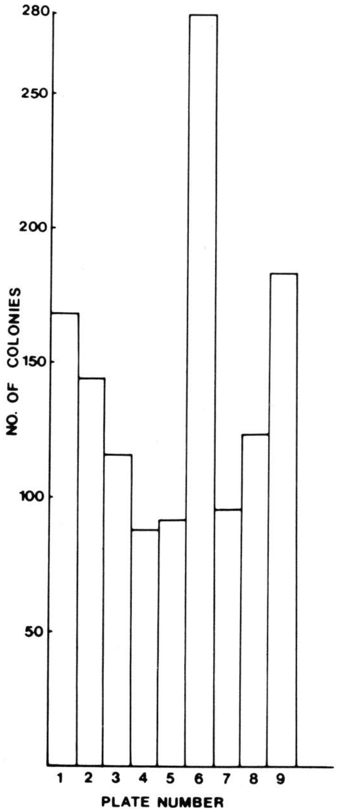

Fig. 2. Quantitative data showing the amount of microbial contamination of agar plates exposed to stream of gas emitted from the laser probe. Ordinate represents number of colony forming units per 120 second duration. Abscissa records sequentially exposed plates.

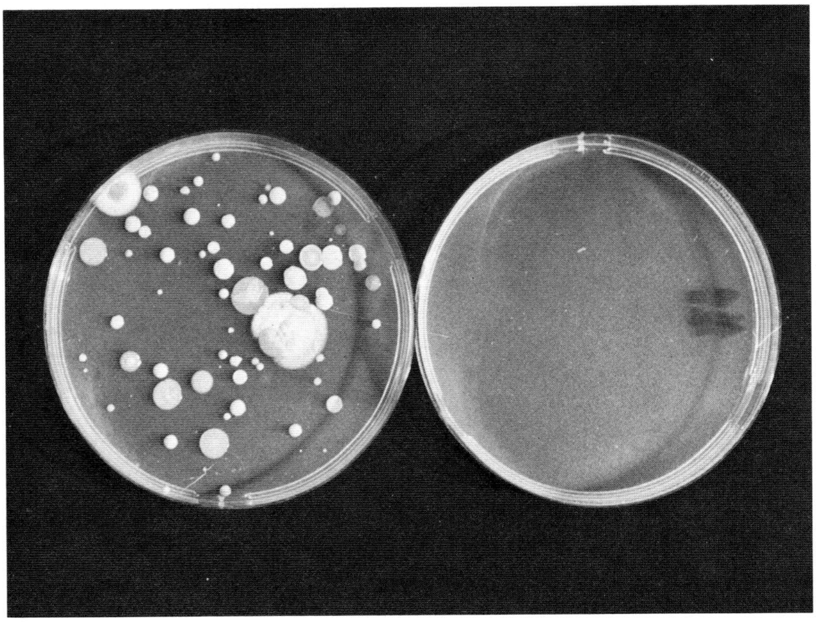

Fig. 3. Typical agar plate exposed to gas emission (left) vs control plate (right).

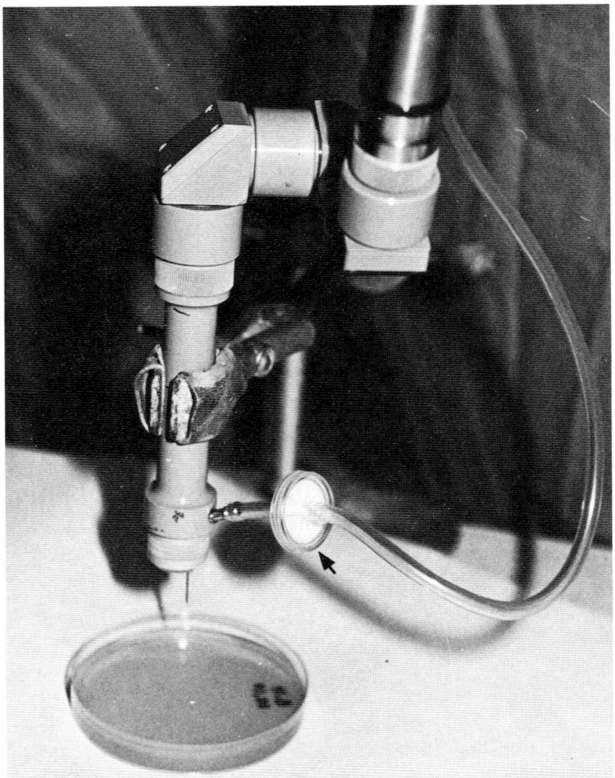

Fig. 4. Insertion of sterile filter on the end of the plastic tube entering the probe (arrow).

of the plates was always found. Prolonged emission of gas did not necessarily reduce the numbers of viable microorganisms emitted with time (Fig. 2). In one experiment (results not shown), 461 colonies of bacteria and fungi were scored on a single plate exposed to the gas emission for a period of 60 seconds.

The colony-forming microorganisms which appeared on the plates probably represent a small fraction of the total number of microbial cells emitted from the probe: (1) Most airborne microorganisms are bound to particulate matter; thus only one colony may result from emission of a single dust particle bearing many cells; (2) many microorganisms are unable to proliferate and form colonies under the growth conditions studied [16]; and (3) the actual contaminated surface is probably much wider than the radius of the agar plate (4.5 cm). Thus, the results presented here represent minimal values.

Contamination of the working surface by a medical laser can pose a threat during surgery. Whereas the laser beam sterilizes the site of intervention, the wound is exposed to at least 3 seconds of contaminating gas stream following the laser irradiation itself, whether continuous or single beam. While it is true that the great majority of the bacterial contaminants observed here are probably not virulent patho-

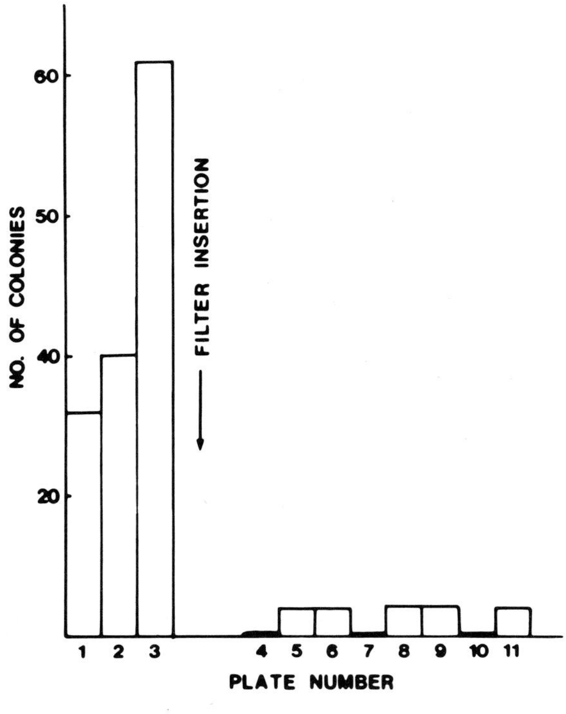

Fig. 5. Quantitative data showing the relative contamination before and after sterilizing measures were taken. The first three plates were sequentially exposed to gas emission as in the legend to Figure 2. At this stage, the filter was inserted, a sterile probe tip was added, and the lens was cleaned with 70% ethanol solution. Negligible contamination was observed in the plates (4–11) sequentially exposed to the gas stream following these measures.

gens, it is well-known that opportunistic microorganisms may proliferate and infect compromised patients [16]. Thus it is our opinion that the contamination observed here is a source of concern.

In order to overcome this problem, the following steps were taken: (1) A sterile filter (transducer protector, Cobe Laboratories, Inc, Lakewood, CO) was inserted on the end of the plastic tube at the entrance to the probe handle (Fig. 4); (2) the plastic tip of the probe handle was sterilized by autoclave; and (3) the laser lens was sterilized with a 70% ethanol solution.

Following these simple procedures, the level of contamination dropped to negligible values (Fig. 5). The authors did not attempt to locate the exact source of the contamination, but it is likely that it originated either in the nitrogen gas supply, or the plastic tubing. In a hospital setting, the tip of the probe may also become contaminated with potentially harmful bacteria or viruses. Since cooling by gas emission is common among medical lasers, we recommend that simple procedures, similar to those described here, be implemented in order to eliminate the possibility of supercontamination of wound sites during use of such lasers in medical settings.

REFERENCES

1. Bailin PC, Ratz JL, Lutz-Nagey L: CO_2 laser modification of Moh's surgery. J Dermatol Surg Oncol 7:621–623, 1981.
2. Kaplan I, Ger J, Sharon V: The carbon dioxide laser in plastic surgery. Br J Plastic Surg 26:359–362, 1973.
3. Klein DR: The use of the carbon dioxide laser in plastic surgery. South Med J 70:429–431, 1977.
4. Labandter H, Kaplan I: Experience with a "continuous" laser in the treatment of suitable cutaneous conditions: A preliminary report. J Dermatol Surg Oncol 3:5, 1977.
5. Peled I, Shohat B, Gassner S, Kaplan I: Excision of epithelial tumors: CO_2 laser versus conventional methods. Cancer Lett 2:41–46, 1976.
6. Beckman H, Fuller TA: Carbon dioxide laser scleral dissection and filtering procedure for glaukoma. Am J Ophthalmol 88:73–77, 1979.
7. Smith MR, Miller JB: New trends in carbon dioxide laser microsurgery. J Microsurg 1:354–363, 1980.
8. Meyer HJ, Vonnalime FJ, Haverkampf K, Huchzermeyer H: Laser coagulation in the upper G.I. tract. A preliminary light and scanning electron microscopic study. Lasers Surg Med 1:103–112, 1980.
9. Slutzki S, Abramshon R, Bogokowsky H: Carbon dioxide laser in the treatment of high anal fistula. Am J Surg 141:395–396, 1981.
10. Onozuka M, Iwata T, Matsuo T, Imai S, Sugaya E, Suzuki Y, Tomita F, Mita S: Decrease of dental enamel solubility following low energy laser irradiation. IRCS Medical Science Biomedical Technology; Connective Tissue, Skin and Bone; Dentistry and Oral Biology 9:1035, 1981.
11. Groenhuis RAJ, Jongebloed WL, Len-Bosh JJ: Surface roughness of acid-etched and demineralized bovine enamel measured by a laser speckle method. Caries Res 14:333–340, 1980.
12. Bjelkhagen H, Sundstrom F, Angmar-Mansson B, Ryden H: Early detection of enamel caries by the luminescence excited by visible laser light. Swed Dent J 6:1–7, 1982.
13. Adrian JC, Gross A: A new method of sterilization—The carbon dioxide laser. J Oral Pathol 8:60–61, 1979.
14. Hooks TW, Adrian JC, Gross A, Bernier WE: Use of the carbon dioxide laser in sterilization of endodontic reamers. Oral Surg 49:263–264, 1980.
15. Stellar S, Polonyi TG, Bredemeir AC: Lasers in surgery. In Wolbarsht ML (ed): "Laser Applications in Medicine and Biology," Vol 2. New York: Plenum Press, 1974, pp 265–268.
16. Rosenberg E, Cohen IR: "Microbial Biology." New York: CBS Publishing, 1983.

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Desorption of adherent bacteria from a solid hydrophobic surface by oil

Mel Rosenberg[1,2,*], Herbert Judes[1] and Ervin Weiss[1]
School of Dental Medicine[1] and Department of Human Microbiology[2], Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv (Israel)

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Summary

Oils efficiently desorbed attached bacteria from a solid test surface (polystyrene). Desorption was measured qualitatively by staining techniques, and quantitatively by following the outgrowth kinetics of cells remaining on the plastic surface following the washing procedure. Approximately 97% of *Acinetobacter calcoaceticus* RAG-1 cells adhering to a polystyrene cuvette were removed following two successive washings in the presence of olive oil. Results suggest that oils may serve as potent, non-toxic desorbing agents to remove attached hydrophobic bacteria from surfaces.

Key words: Acinetobacter – *Adherence – Desorption – Hydrophobicity – Oils – Polystyrene*

In recent years, hydrophobic interactions have emerged as a major factor in mediating bacterial adherence, both in the open environment [1–4], as well as in human and other hosts [5–8]. *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012), a hydrocarbon-degrading bacterium, has proved a useful model microorganism in the study of various adherence phenomena. RAG-1 adheres avidly to a variety of hydrophobic surfaces, including liquid hydrocarbons [4, 9–11], polystyrene [9, 12], as well as to human buccal epithelial cells [11, 13] and teeth (M. Rosenberg, H. Judes and E. Weiss, submitted for publication). A nonhydrophobic mutant derived from RAG-1 [4] is deficient in adherence to all the above surfaces.

Although many investigators have studied the processes by which bacteria become attached to surfaces [3, 14], relatively little research has been devoted to the removal of bacteria which are already bound. Desorption of adherent microorganisms from environmental biofilms and human tissues constitutes a problem of

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[*] To whom reprint requests should be addressed at the School of Dental Medicine.

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wide environmental [3] and medical [5, 11, 13] interest. The ability of an amphipathic polymer to prevent and reverse adherence of bacteria (including RAG-1) to hydrocarbons and epithelial cells has recently been reported [13]. In the present study we demonstrate that the high affinity of RAG-1 cells for oil enables their efficient desorption from the hydrophobic test surface, polystyrene.

The affinity of RAG-1 for various oils is compared in Table 1. Among the various edible oils tested, purified olive and corn oil bound the highest proportions of RAG-1 cells. In subsequent experiments described here, olive oil and hexadecane were chosen as test oil and test hydrocarbon, respectively, in studying bacterial desorption.

Desorption was studied by simple techniques: RAG-1 cells were grown for 18 h to stationary phase in brain heart infusion broth (Difco), conditions which optimize their adherence characteristics [4]. Cells were harvested, washed twice and suspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_2O$; 7.26 KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml, pH 7.1), to a turbidity of ca. 250 Klett units (measured in a Klett Summerson colorimeter fitted with a green filter). In order to obtain a layer of bound cells, 1.5 ml cell suspension was mixed by vortex for 60 s within standard polystyrene cuvettes (Rudolph Brand, Wertheim/Main, FRG). The cell suspension was then poured from the cuvettes. Each cuvette was then washed with 1.5 ml PUM to remove non-bound or poorly attached cells.

To 1.5 ml PUM in each cell-coated cuvette, 0.3 ml olive oil or hexadecane was added, and the mixtures were agitated for 120 s by hand. The mixtures were poured off and the process was repeated. Following the second mixing, cuvettes were rinsed 4 times with 1.5 ml PUM to remove as much of the non-aqueous phase as possible from the cuvettes. Control cuvettes were subjected to exactly the same

TABLE 1

BINDING OF RAG-1 BY OILS

RAG-1 cells, grown for 18 h on brain heart infusion broth were washed twice and suspended in PUM to an initial turbidity at 650 nm of 1.07. Adherence to oils was performed by a slight modification of the previously described method for adherence to liquid hydrocarbons [10]. To acid-washed test tubes containing 2 ml aqueous suspension of cells, 0.1 ml oil was added and then mixed by vortex for 120 s. Turbidity of the lower aqueous phase was recorded at 650 nm and compared with that of the original suspension. Results are expressed as the percentage decrease in cell-mediated turbidity following the assay, as compared with the original suspension. Oils were obtained from Sigma; *n*-hexadecane was obtained from Fluka.

Oil	Percent bacteria removed from aqueous phase
Bay	60
Safflower seed	69
Coconut	70
Soybean	81
Corn	87
Olive	87
n-Hexadecane	83

treatment, except that the mixing was performed with 1.5 ml PUM, in the absence of oil.

In order to study desorption of cells qualitatively, cuvettes were fixed with methanol and stained with Gentian Violet [12]. Control cuvettes which were washed in the absence of oil maintained a dense layer of attached RAG-1 cells (Fig. 1), despite the repeated mixings in the presence of buffer. However, cuvettes washed in the presence of either olive oil or hexadecane appeared completely free of bound cells.

To obtain a quantitative estimate of the number of remaining adherent cells, 1.2 ml of minimal medium [4], consisting of PUM supplemented with 0.2% sodium acetate as carbon and energy source, was added to each cuvette. Cuvettes were incubated horizontally at 30°C. Growth was followed turbidimetrically directly in the cuvettes at 400 nm using a Uvikon 710 spectrophotometer (Kontron, Zurich, Switzerland).

Results of a typical experiment are shown in Fig. 2. In the control cuvette, exponential growth was observed following ca. 3 h of incubation. In the cuvette washed with olive oil, growth commenced after 6 h. In both cases doubling time was observed to be the same (50 min). Since a 1:200 dilution of the RAG-1 cells used in the experiment inoculated into the minimal medium and incubated in cuvettes under the same conditions grew following a lag of 100 min (not shown), back extrapolation of the growth curves to 100 minutes provided an estimation of the initial bacterial populations. The extrapolated initial absorbance value obtained

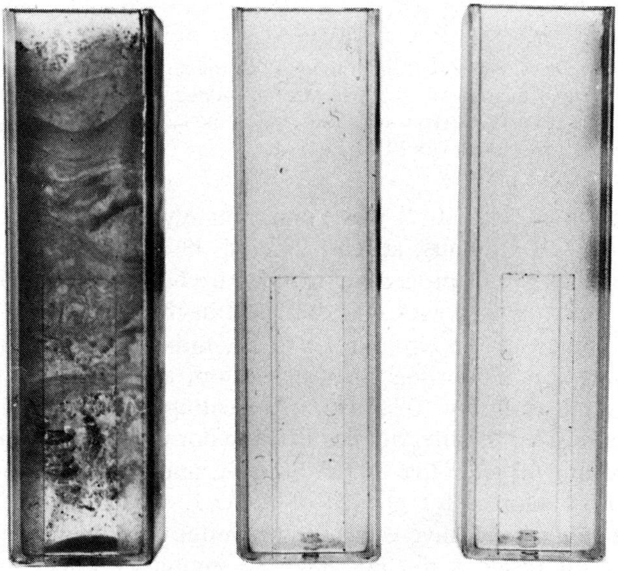

Fig. 1. Cuvettes were washed and rinsed, as described in the text. The remaining adherent cells were visualized by fixation in methanol and staining with Gentian Violet. Left, control cuvette, washed with PUM alone; center, cuvette washed with olive oil; right, cuvette washed with *n*-hexadecane.

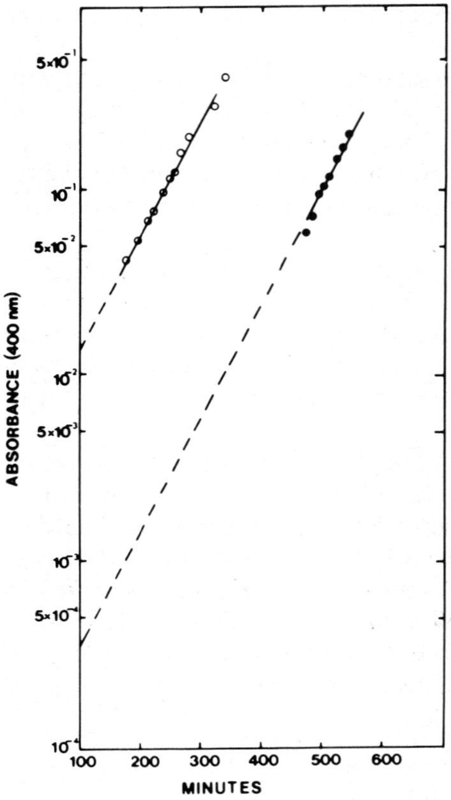

Fig. 2. RAG-1 cells bound to polystyrene were desorbed and rinsed as described in the text. To each cuvette, 1.2 ml acetate medium (PUM supplemented with 0.2% acetate) was added. Outgrowth of cells was measured turbidimetrically. Results are back extrapolated to the end of the lag period (100 min). (●) washed with olive oil:PUM mixture; (○) washed with PUM alone.

for the olive-oil washed cuvette (3.5×10^{-4}) was approximately 2.5% of that observed for the control (1.4×10^{-2}). Thus, at least 97% of the bound RAG-1 cells was removed from the cuvette by this procedure, as compared with the control washed in PUM alone. No growth whatsoever was observed in the hexadecane-washed cuvettes during the course of the experiment (9 h), indicating an even higher level of bacterial desorption. Following 20 h incubation, all the cuvettes reached approximately the same turbidity (0.95–1.07), indicating an equivalent growth yield. It should be emphasized that neither hexadecane nor olive oil is toxic for RAG-1 cells; both hexadecane [4] and olive oil (Y. Shabtai, unpublished data) can serve as carbon and energy sources for growth of RAG-1. The equivalent growth rates measured in the control and olive-oil washed cuvettes provide further evidence that any remaining oil residues did not have an inhibitory effect on growth.

The data demonstrate that oils can efficiently desorb bacteria adhering to a hydrophobic test surface. The non-toxic nature of edible oils suggests their potential use as bacterial desorption agents within the human body. Of particular interest is the possibility that edible oils may be able to desorb attached bacteria from the oral cavity. Recent studies have attested to the high proportion of hydrophobic microbial flora on the tooth surface (ref. 8 and M. Rosenberg, H. Judes and E. Weiss, manuscript submitted) as well as on buccal epithelial cells [13].

The use of outgrowth experiments to study bacterial desorption provides a simple quantitative means of verifying desorption as measured by qualitative (e.g. staining) techniques. In addition, it may provide information on other effects, such as inhibition or stimulation of growth rate of cells following the desorption treatment.

The use of polystyrene as a model surface for studies involving bacterial adherence and desorption has several advantages: (i) optically clear, smooth, uniform polystyrene surfaces are readily available to the microbiologist in various forms (plate, cuvette, test tube, etc.); (ii) attached bacterial layers are easily attained [12]; (iii) polystyrene has been successfully employed as a substratum for attachment of various bacteria [2, 12, 15]; (iv) the hydrophobic interactions which mediate bacterial adherence to polystyrene may similarly mediate bacterial adherence to a wide variety of surfaces of ecological and medical interest, including the water:air interface [1], phagocytes [7], human buccal epithelial cells [5, 11, 13], and teeth (ref. 8 and M. Rosenberg, H. Judes and E. Weiss, submitted for publication).

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References

1 Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. (1981) The hydrophobicity of bacteria – an important factor in their initial adhesion at the air-water interface. Arch. Microbiol. 128, 267-270.
2 Fletcher, M. and Loeb, G.I. (1979) Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. 37, 67-72.
3 Marshall, K.C. (1976) Interfaces in Microbial Ecology. Harvard University Press, Cambridge, MA.
4 Rosenberg, M. and Rosenberg, E. (1981) Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. 148, 51-57.
5 Ofek, I., Simpson, W.A. and Beachey, E.H. (1982) Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. J. Bacteriol. 149, 426-433.
6 Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, T. (1978) Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. 22, 462-472.

7 Van Oss, C.J. (1978) Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. 32, 19-40.

8 Weiss, E., Rosenberg, M., Judes, H. and Rosenberg, E. (1982) Cell surface hydrophobicity of adherent oral bacteria. Curr. Microbiol. 7, 125-128.

9 Rosenberg, M., Bayer, E.A., Delarea, J. and Rosenberg, E. (1982) Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. Appl. Environ. Microbiol. 44, 929-937.

10 Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

11 Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. 33, 29-33.

12 Rosenberg, M. (1981) Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. 42, 375-377.

13 Rosenberg, E., Gottlieb, A. and Rosenberg, M. (1983) Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. Infect. Immun. 39, 1024-1028.

14 Characklis, W.G. (1981) Fouling biofilm development: a process analysis. Biotechnol. Bioeng. 23, 1923-1960.

15 Gerson, D.F. and Scheer, D. (1980) Cell surface energy, contact angles and phase partition. III. Adhesion of bacterial cells to hydrophobic surfaces. Biochim. Biophys. Acta 602, 506-510.

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NOTES

Cell Surface Hydrophobicity of Dental Plaque Microorganisms In Situ

MEL ROSENBERG,[1,2]* HERBERT JUDES,[1] AND ERVIN WEISS[1]

School of Dental Medicine[1] *and Department of Human Microbiology,*[2] *Sackler Faculty of Medicine, Tel Aviv University, Ramat-Aviv, Israel*

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The cell surface hydrophobicity of bacteria obtained directly from human tooth surfaces was assayed by measuring their adherence to liquid hydrocarbons. Fresh samples of supragingival dental plaque were washed and dispersed in buffer. Adherence of the plaque microorganisms to hexadecane, octane, and xylene was tested turbidimetrically and by direct microscopic observation. The results clearly show that the vast majority of bacteria comprising dental plaque exhibit pronounced cell surface hydrophobicity. These data support the hypothesis that hydrophobic interactions play a major role in mediating bacterial adherence on tooth surfaces.

The role of cell surface hydrophobicity in mediating bacterial adherence to mammalian cells was conceived of over half a century ago in the pioneering studies of Mudd and Mudd (11, 12). During the past decade, the development of quantitative techniques for the study of bacterial hydrophobicity (2–5, 10, 16, 20, 25, 26) has facilitated studies on the role of hydrophobic interactions in the adherence and subsequent proliferation of bacteria on solid surfaces and at liquid-liquid and liquid-air interfaces. Bacterial hydrophobicity has been implicated in phagocytosis (26), partitioning of bacteria at the air-water interface (2), adherence and growth on hydrocarbon (10, 19, 22), attachment of bacteria to buccal epithelial and intestinal mucosal cells (16, 17, 21, 25), adherence of bacteria to nonwettable plastics (4, 5, 18), and adherence of benthic cyanobacteria to sediment (24).

In a recent study (27), the hydrophobic properties of 102 adherent bacterial isolates obtained from the surfaces of freshly extracted teeth were determined on the basis of their affinity for liquid hydrocarbon (hexadecane). An extremely high proportion (80%) of the isolates tested adhered with high affinity at the aqueous-hydrocarbon interface. Similarly, 58% of the isolates obtained from the surfaces of stainless steel dental matrices incubated briefly in the oral cavity were hydrophobic. This investigation presented initial experimental data in support of the premise that the majority of plaque microorganisms are hydrophobic and that hydrophobic interactions are involved in mediating adherence of oral bacteria to human tooth surfaces. However, two objections could be raised in this regard: (i) the hydrophobic surface properties observed after growth of the isolates in vitro do not necessarily reflect the surface characteristics of the same bacteria on tooth surfaces (6); it is known that cell surface hydrophobicity may change as a function of the physiological state of the bacteria (21); and (ii) since the great majority of bacteria on tooth surfaces are not cultivable (14), the isolates tested represent a small, disproportionate sample of the microbial population extant on human teeth.

In the present investigation we attempted to overcome these possible reservations by investigating the hydrophobic surface properties of microorganisms obtained directly from tooth surfaces in the form of supragingival dental plaque. Supragingival dental plaque is a macroscopic biofilm (1), consisting of a dense, heterogeneous array of bacteria directly or indirectly attached to tooth surfaces (14), which has been directly linked to oral disease in human volunteers (9). In the experiments described here, supragingival dental plaque was scraped with a sterile curette from the mouths of healthy adult volunteers and dispersed in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3 H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7 H_2O$, and distilled water to 1,000 ml [pH 7.1]) by mild shearing for 2 min in an Ultra-turrax homogenizer (Janke and Kunkel Ika-Werk, Staufe, Federal Republic of

TABLE 1. Adherence of dispersed plaque to hydrocarbons[a]

Test hydrocarbon	% Adherence in sample[b]:							% Mean adherence
	1	2	3	4	5	6	7	
n-Hexadecane	62	76	85	68	61	60	62	68
n-Octane	80	94	100	100	83	91	100	93
p-Xylene	87	99	100	99	100	100	95	97

[a] Supragingival dental plaque was collected from seven individuals, washed, and dispersed in PUM buffer to yield an initial absorbance of 0.8 to 1.0 at 400 nm. To 1.2 ml of dispersed plaque, 0.3 ml of hydrocarbon was added, and the solution was mixed uniformly for 120 s. After phase separation, the lower aqueous phase was transferred to a fresh test tube, an additional 0.3 ml of hydrocarbon was added, and the solution was mixed as described above. After this second extraction, the lower aqueous phase was transferred to a semi-micro cuvette, and its absorbance was recorded.

[b] Results are presented as the percent decrease in absorbance compared with that of the corresponding control mixed without added hydrocarbon.

Germany). The dispersed plaque suspension was washed by centrifugation and suspended in PUM buffer by dispersion (as above) to yield an absorbance at 400 nm of 0.8 to 1.0, as measured with a Uvikon 710 spectrophotometer (Kontron, Zurich, Switzerland). Phase microscopic observation verified that the dispersed, washed plaque consisted mainly of single, intact bacterial cells.

The basic technique for measuring bacterial adherence to liquid hydrocarbons (19–23) was employed in this study with the following modifications. To 1.2 ml of dispersed plaque in round-bottom test tubes (12 by 75 mm) 0.3 ml of test hydrocarbon (*n*-hexadecane, *n*-octane, or *p*-xylene) was added, and the solutions were mixed uniformly for 120 s. After 10 min for phase separation, the lower aqueous phase was transferred to fresh test tubes, each containing 0.3 ml of the same test hydrocarbon. They were mixed as described above. After this second extraction, the lower aqueous phase was removed into semi-micro cuvettes (Greinier, Nurtingen, Federal Republic of Germany), and absorbance at 400 nm was measured. The results are expressed as the percent decrease in turbidity as compared with that of the corresponding sample mixed without added hydrocarbon. The turbidimetric results were corroborated by phase microscopic examination of the aqueous phases before and after mixing, as well as observation of the hydrocarbon droplets after the assay. In all cases, the drop in turbidity corresponded with the disappearance of bacteria from the lower aqueous phase and their appearance on the surface of hydrocarbon droplets which rose after the mixing procedure (20).

The results of a typical experiment, in which adherence of dispersed plaque obtained from seven volunteers was measured, are shown in Table 1. All three test hydrocarbons were able to bind the majority of the dispersed plaque bacteria in all seven samples. Optimal adherence was observed in the presence of xylene; on the average, a 97% decrease in cell-mediated turbidity was observed. Cells also bound to octane with extremely high affinity. Adherence to hexadecane was lower than that observed for the other two hydrocarbons. This sequence of affinity for the test hydrocarbons has been observed with nonoral laboratory strains (23) and may be related to the relatively high viscosity of hexadecane. Results similar to those presented in Table 1 were obtained in experiments in which the plaque was dispersed by sonic oscillation and then suspended and washed in saline or phosphate-buffered saline. In experiments employing plaque which was dispersed in PUM buffer but not washed before the adherence assay, the percentage of adherent cells was ca. 20% lower than that shown in Table 1. This is probably due to the presence of amphipathic molecules in the unwashed samples which interfere with the adherence assay (17).

Figure 1 illustrates the adherence of plaque bacteria to octane droplets after the assay procedure. Various morphological forms, including diplococci, filaments, fusiforms, spiral forms, and large rods, all typical of plaque microbiota (14), were readily observed adhering at the oil-water interface, whereas the bulk aqueous phase was almost completely devoid of bacteria.

The data presented here demonstrate that the large majority of bacteria comprising supragingival plaque exhibit pronounced cell-surface hydrophobicity in situ, as evidenced by their avid affinity for liquid hydrocarbons (20). The relatively small differences observed among the plaque samples obtained from different individuals attest to the general nature of this phenomenon. The results support the hypothesis that hydrophobic interactions play a major role in bacterial adherence on human tooth surfaces and subsequent plaque accumulation. Other observations which support this premise include the low surface energy found for the acquired

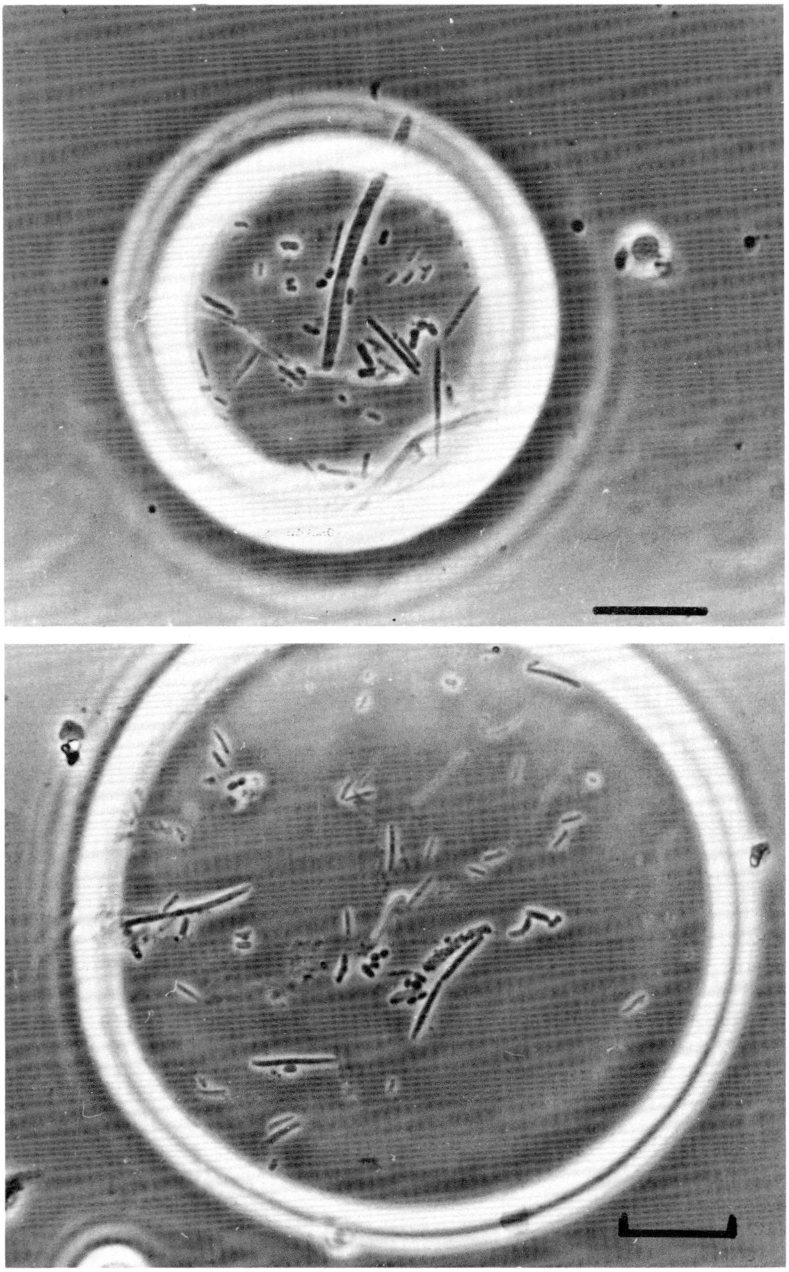

FIG. 1. Adherence of dispersed dental plaque to octane droplets. Supragingival dental plaque obtained from one individual was dispersed by shearing and washed as described in the text. After being mixed in the presence of octane as described in footnote *a* of Table 1, plaque bacteria became concentrated at the oil-water interface. Phase microscopic observation of droplets revealed adherence of various plaque microorganisms, with the surrounding aqueous phase almost completely free of bacteria. Bars, 10 μm.

pellicle which covers tooth enamel in situ (7, 8), the hydrophobic nature of many cultivable oral strains, and correlations between bacterial hydrophobicity and adherence to saliva-coated hydroxyapatite (13, 15, 27; R. J. Gibbons and I. Etherden, Infect. Immun., in press). The high cell surface hydrophobicity of plaque microorganisms, as presented here, may conceivably be due in part to salivary components which are adsorbed on the bacterial cell surfaces in situ. However, preliminary data (I. Eli and M. Rosenberg, unpublished results) indicate that saliva acts to inhibit, rather than promote, bacterial adherence to hydrophobic surfaces. Experiments are under way to further investigate the relationship between bacterial hydrophobicity and adherence within the oral cavity.

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LITERATURE CITED

1. **Characklis, W. G.** 1981. Fouling biolfilm development: a process analysis. Biotechnol. Bioeng. **23**:1923–1960.
2. **Dahlback, B., M. Hermansson, S. Kjelleberg, and B. Norkrans.** 1981. The hydrophobicity of bacteria—an important factor in their initial adhesion at the air-water interface. Arch. Microbiol. **128**:267–270.
3. **Doyle, R. J., W. E. Nesbitt, and K. G. Taylor.** 1982. On the mechanism of adherence of *Streptococcus sanguis* to hydroxylapatite. FEMS Microbiol. Lett. **15**:1–5.
4. **Fletcher, M., and G. I. Loeb.** 1979. Influence of substratum characteristics on the attachment of a marine pseudomanad to solid surfaces. Appl. Environ. Microbiol. **37**:67–72.
5. **Gerson, D. F., and D. Scheer.** 1980. Cell surface energy, contact angles and phase partition. III. Adhesion of bacterial cells to hydrophobic surfaces. Biochim. Biophys. Acta **602**:506–510.
6. **Gibbons, R. J.** 1980. Adhesion of bacteria to the surfaces of the mouth, p. 351–388. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Adsorption of microorganisms to surfaces. Ellis Horwood, Chichester, England.
7. **Glantz, P.-O.** 1969. On wettability and adhesiveness. A study of enamel, dentine, some restorative materials, and dental plaque. Odontol. Rev. **20**(Suppl. 17):1–132.
8. **Glantz, P. O.** 1977. Adhesion to teeth. Int. Dent. J. **27**:324–332.
9. **Löe, H., E. Theilade, and S. B. Jensen.** 1965. Experimental gingivitis in man. J. Periodontol. **36**:177–187.
10. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass.
11. **Mudd, S., and E. B. H. Mudd.** 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. **40**:633–645.
12. **Mudd, S., and E. B. H. Mudd.** 1924. Certain interfacial tension relations and the behaviour of bacteria in films. J. Exp. Med. **40**:647–660.
13. **Nesbitt, W. E., R. J. Doyle, and K. G. Taylor.** 1982. Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite. Infect. Immun. **38**:637–644.
14. **Nolte, W. A.** 1982. Oral Microflora, p. 193–228 *In* W. A. Nolte (ed.), Oral microbiology. C. V. Mosby Co., St. Louis.
15. **Olsson, J., and G. Westergren.** 1982. Hydrophobic properties of oral streptococci. FEMS Microbiol. Lett. **15**:319–323.
16. **Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tagesson.** 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Pathol. Microbiol. Scand. Sect. B **85**:308–316.
17. **Rosenberg, E., A. Gottlieb, and M. Rosenberg.** 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect. Immun. **39**:1024–1028.
18. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42**:375–377.
19. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. Appl. Environ. Microbiol. **44**:929–937.
20. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.
21. **Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg, and I. Ofek.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. **33**:29–33.
22. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane. J. Bacteriol. **148**:51–57.
23. **Rosenberg, M., S. Rottem, and E. Rosenberg.** 1982. Cell surface hydrophobicity of smooth and rough **Proteus mirabilis** strains as determined by adherence to hydrocarbons. FEMS Microbiol. Lett. **13**:167–169.
24. **Shilo, M., and A. Fattom.** 1983. The ecology and adaptive strategies of benthic cyanobacteria. Dundee Symposium, Sept. 7–9, 1982, Society for General Microbiology.
25. **Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22**:462–472.
26. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32**:19–40.
27. **Weiss, E., M. Rosenberg, H. Judes, and E. Rosenberg.** 1982. Cell surface hydrophobicity of adherent oral bacteria. Curr. Microbiol. **7**:125–128.

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Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbon

Eugene Rosenberg *, Nachum Kaplan, Ophry Pines, Mel Rosenberg and David Gutnick

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv, Israel

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1. INTRODUCTION

Growth of *Acinetobacter calcoaceticus* RAG-1 on hydrocarbons is accompanied by two readily observable phenomena: emulsification of the substrate and adherence of the bacteria to hydrocarbon droplets. Emulsification is due to the production of an extracellular galactosamine-aminouronic polysaccharide, referred to as emulsan [1,2]. Adherence is due, at least in part, to the presence of thin (approx. 3 nm diameter) fimbriae [3]. During early stages of the growth cycle, emulsan accumulates on the surface of RAG-1 in the form of a minicapsule; emulsan is released into the medium during unbalanced growth [4] or when cells approach stationary phase [5]. Preliminary experiments indicating that emulsan release from the cell-surface coincides with an increase in cell surface hydrophobicity led us to examine more directly the relationship between capsular polysaccharide and adherence to hydrocarbons in two *Acinetobacter* strains. In the present study we demonstrate that both the capsule of strain RAG-1 and the rhamnose-glucose capsule of strain BD4 [6] interfere with adherence to hydrocarbons, and thus decrease cell surface hydrophobicity.

2. MATERIALS AND METHODS

A. calcoaceticus RAG-1 (ATCC31012) and an emulsan-deficient mutant, TR3 [7], were grown at 30°C with aeration in EM medium that contained per liter of deionized water: 22.2 g $K_2HPO_4 \cdot 3\ H_2O$; 7.26 g KH_2PO_4; 4.0 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4 \cdot 7\ H_2O$, and 5.0 ml ethanol. For comparing the adherence of strains RAG-1 and TR3, cells were harvested in exponential phase (180 klett units, Klett-Summerson colorimeter fitted with a green filter), harvested, washed twice and suspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3\ H_2O$; 7.26 KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7\ H_2O$, and distilled water to 100 ml).

A. calcoaceticus BD4 [6] and a minicapsule mutant, BD413 trp E27, derived from BD4 [8], were grown at 30°C with aeration in GM medium which contained per liter of deionized water: 5.0 g glucose, 9.17 g $K_2HPO_4 \cdot 3\ H_2O$, 3.0 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$, and 0.2 g $MgSO_4 \cdot 7\ H_2O$, final pH 7.0. The GM medium was supplemented with 100 μg/ml tryptophan for growth of strain BD413 trp E27.

Removal of the large rhamnose-glucose capsule of strain BD4 was carried out by incubating washed cells (250 klett units, green filter) with a BD4 capsule-degrading enzyme preparation in distilled water for 16 h at 30°C. The source of the enzyme preparation was a clarified lysate of *Acinetobacter* bacteriophage SL-1 (N. Kaplan, unpublished data).

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* To whom reprint requests should be addressed.

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After centrifugation at 17 300 × g for 20 min at 4°C, the enzyme was precipitated from the supernatant fluid by addition of $(NH_4)_2SO_4$ to 40% saturation. The pellet was suspended in distilled water and residual phages were removed by centrifugation at 140 000 × g for 2 h at 4°C.

The amount of cell-bound and cell-free rhamnose-containing BD4 polysaccharide was determined by the cysteine-sulfuric acid reaction [9], as previously described [10]. The amount of emulsan on the cell surface of RAG-1 and TR3 cells was determined from the polymeric galactosamine content of the cells as described previously [4].

The technique for measuring adherence of bacteria to hydrocarbons has been previously described [11]. Cells were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of approx. 1.5. Various volumes of *n*-hexadecane were added to round-bottom acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed-cell suspension, and the phases were vortexed uniformly for 120 s. After 15 min during which time the phases were allowed to separate, the turbidity of the lower aqueous phase of each tube was measured at 400 nm. Data are presented either as the % absorbance relative to that of the initial cell suspension, or as % adherence which is the % decrease in absorbance.

3. RESULTS AND DISCUSSION

Although *A. calcoaceticus* RAG-1 adheres avidly to hydrocarbons following growth on complex or hydrocarbon media [12], cells were poorly adherent when harvested from exponential phase on EM medium (Fig. 1). Since growth of RAG-1 on EM medium led to the accumulation of an emulsan capsule (approx. 20% of the cell dry weight), it was of interest to examine the adherence of the emulsan-deficient mutant TR3. When grown under identical conditions, 70% of the TR3 cells were removed from the aqueous phase by 0.2 ml hexadecane, whereas only 20% of the RAG-1 cells were extracted (Fig. 1).

The hypothesis that polysaccharide capsules of *Acinetobacter* inhibit adherence to hydrocarbon was further tested with strain BD4 and its mini-

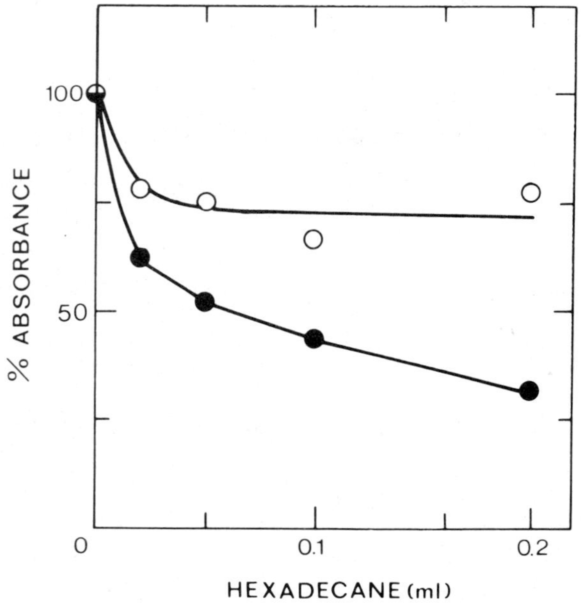

Fig. 1. Adherence of exponential phase *A. calcoaceticus* RAG-1 (○) and TR3 (●) to hexadecane.

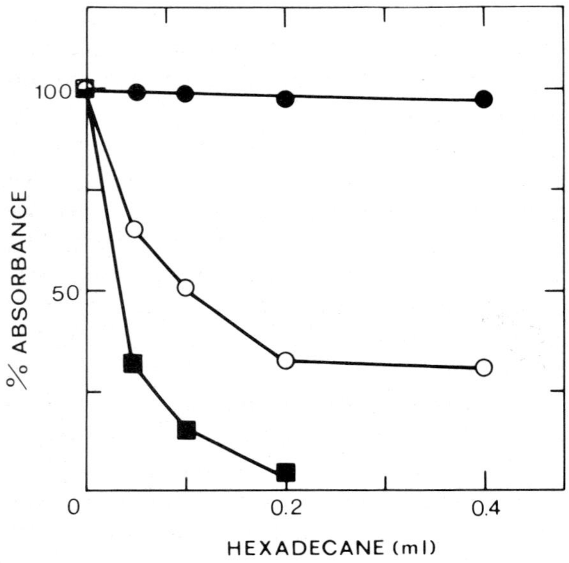

Fig. 2. Adherence of *A. calcoaceticus* BD4 (●), BD413 (○), and enzymatically decapsulated BD4 (■) to hexadecane. The amounts of cell-bound polymeric rhamnose/mg cell dry weight were 202 μg (BD4), 35 μg (BD413) and 10 μg (decapsulated BD4).

capsulated mutant BD413 (Fig. 2). Wild-type BD4 cells contained a large rhamnose-glucose capsule (27% of cell dry weight) and did not adhere at all to the test hydrocarbon. The minicapsulated mutant BD413 (capsule comprised 11% of cell dry weight) adhered to the extent of 70% in the presence of 0.2 ml hexadecane. The enzymatically decapsulated BD4 cells contained 5% of the initial capsular rhamnose (decapsulation was also confirmed by India ink staining) and were 98% adherent. The quantitative relationship between BD4 capsule content and adherence is shown in Fig. 3. Approx. 75% of the capsule was removed enzymatically without any significant increase in adherence. However, further decrease in capsule content resulted in a dramatic increase in the ability of the cells to adhere to hexadecane. When 90% and 95% of the capsule was removed, the cells adhered to the extent of 72 and 98%, respectively.

The comparison of two sets of mutant strains with their corresponding parent strains and the enzymatic decapsulation study clearly demonstrate that polysaccharide capsules can decrease *A. calcoaceticus* adherence to hydrocarbon, presumably by rendering the outer bacterial surface more hydrophilic. The relevance of this conclusion to growth of *Acinetobacter* strains on hydrocarbons is not clear. Adherence is an important factor in initiating growth on hexadecane [12]. Starved cells with little or no capsule might adhere more tightly to hydrocarbon substrata. As growth proceeds and the hydrocarbon–water interface becomes crowded, desorption may be a necessary first step in colonizing fresh interfaces. Desorption could be brought about by the synthesis of capsule; thus, the ability of cells to regulate the extent of their capsule may be advantageous in changing microenvironments.

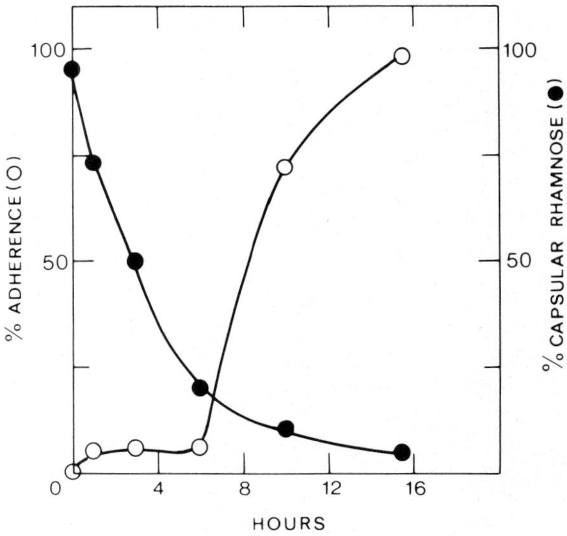

Fig. 3. Adherence of *A. calcoaceticus* BD4 as a function of rhamnose capsule. Cells were treated with the phage capsule depolymerase for various times, harvested, washed and assayed for rhamnose capsule (presented as % of total rhamnose exopolysaccharide) and adherence to 0.2 ml hexadecane.

Cell surface hydrophobicity has been cited as an important factor in determining phagocytosis of bacterial cells [13]. The data presented here indicating the increased hydrophobicity of decapsulated *Acinetobacter* strains are consistent with, and help explain the well-documented observation that polysaccharide capsules assist bacteria in evading phagocytosis [14]. Recently, it has been shown that the hyaluronic acid capsule of *Streptococcus pyogenes* similarly prevent adherence of these cells to hexadecane (I. Ofek, E. Whitnack and E.H. Beachey, unpublished data). Studies are underway to further investigate the role of cell-surface and extracellular polysaccharides in the growth of *Acinetobacter calcoaceticus* on hydrocarbons.

REFERENCES

[1] Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. (1979) Appl. Environ. Microbiol. 37, 402–408.

[2] Zuckerberg, A., Diver, A., Peeri, Z., Gutnick, D.L. and Rosenberg, E. (1979) Appl. Environ. Microbiol. 37, 414–420.

[3] Rosenberg, M., Bayer, E.A., Delarea, J., and Rosenberg, E. (1982) Appl. Environ. Microbiol. 44, 929–937.

[4] Rubinovitz, C., Gutnick, D.L. and Rosenberg, E. (1982) J. Bacteriol. 152, 126–132.

[5] Goldman, S., Shabtai, Y., Rubinovitz, C., Rosenberg, E. and Gutnick, D.L. (1982) Appl. Environ. Microbiol. 44, 165–170.

[6] Taylor, W.H. and Juni, E. (1961). J. Bacteriol. 81, 688–693.

[7] Pines, O. and Gutnick, D.L. (1981) Arch. Microbiol. 130, 129–133.

[8] Juni, E. (1972) J. Bacteriol. 112, 917–931.

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Inhibition of Bacterial Adherence to Hydrocarbons and Epithelial Cells by Emulsan

EUGENE ROSENBERG,[1]* ANITA GOTTLIEB,[1] AND MEL ROSENBERG[2]

Department of Microbiology, George S. Wise Faculty of Life Sciences,[1] and School of Dental Medicine,[2] Tel-Aviv University, Ramat Aviv, Israel

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Acinetobacter calcoaceticus RAG-1 and BD413, as well as *Streptococcus pyogenes* M-5, adhered to octane. Adherence was inhibited by emulsan (100 μg/ml), the polymeric emulsifying agent produced by *A. calcoaceticus* RAG-1. Emulsan also inhibited adherence of *S. pyogenes* and RAG-1 to buccal epithelial cells. The mean values of bound *S. pyogenes* per epithelial cell were 57.2 and 20.7 for the control and emulsan-containing suspensions, respectively; mean values of bound RAG-1 per epithelial cell were 221 for the control and 40 for the suspension containing 100 μg of emulsan per ml. Desorption of previously bound RAG-1 from epithelial cells by emulsan was concentration dependent: a maximum of 80% desorption was obtained with 200 μg of emulsan per ml. The data showing that emulsan desorbed 70% of the indigenous bacterial flora from buccal epithelial cells suggest that hydrophobic interactions mediate not only the in vitro adherence of laboratory strains to epithelial cells, but actually govern the adherence of the majority of the bacteria that colonize this surface. The advantages of using emulsan as an antiadherence agent include its chemical purity, stability, and polymeric nature.

In many cases, adherence of microorganisms to tissues within the host is a prerequisite for subsequent colonization (5) and thus an essential factor in the disease process. Several different mechanisms have been shown to be involved in adhesion of bacteria to mammalian cells. One such mechanism, hydrophobic interactions, has been reported to be involved in the adherence of enteropathogenic *Escherichia coli* (3, 15, 18), rough strains of *Salmonella typhimurium* (7), and streptococci (1, 6, 11, 16) to mammalian cells. Hydrophobic interactions may also play a role in the adherence of certain oral species to the tooth surface (18a). Cell surface hydrophobicity has also been reported to be an important factor in phagocytosis (17).

Bacterial adherence to liquid hydrocarbons is a general method for measuring cell surface hydrophobicity (9–14). Recently, this technique was employed to demonstrate correlations between the ability of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 and the oral pathogen *Streptococcus pyogenes* M-5 to adhere to buccal epithelial cells and hydrocarbons (11). *A. calcoaceticus* RAG-1 mutant MR-481, selected for its inability to adhere to hydrocarbon (12), also failed to adhere to epithelial cells.

A. calcoaceticus RAG-1 produces a potent extracellular bioemulsifier referred to as emulsan (8). Emulsan is a polyanionic heteropolysaccharide with a molecular weight average of 9.9×10^5 (20). Emulsan consists of a D-galactosamine-containing polysaccharide backbone with covalently linked fatty acid side chains (2, 20). Protein (approximately 15%) associated with emulsan can be removed by hot phenol treatment or calcium nitrate precipitation without destroying its emulsifying activity. Emulsan stabilizes a wide variety of hydrocarbon-in-water emulsions by forming a strong film at the interface (19).

The high affinity of emulsan for hydrocarbon-water interfaces (19) suggested that it might be useful for studying the role of hydrophobic interactions in bacterial adherence phenomena. In this report, data are presented which demonstrate that emulsan prevents adherence of *A. calcoaceticus* and *S. pyogenes* to hydrocarbon and buccal epithelial cells. Emulsan was also shown to be highly effective in removing bound indigenous bacteria from epithelial cells.

MATERIALS AND METHODS

Bacterial growth conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) was grown in nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl and harvested in stationary phase after overnight incubation at 30°C with shaking. For adherence to octane, the cells were then diluted 1:100 into fresh

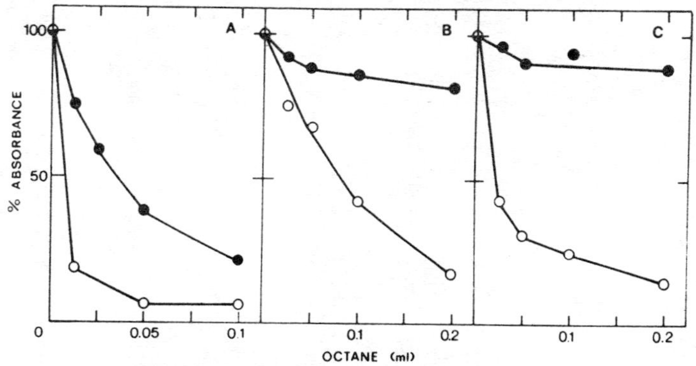

FIG. 1. Adherence of *A. calcoaceticus* RAG-1 (A), *A. calcoaceticus* BD413 (B), and *S. pyogenes* M-5 (C) to octane. Washed cells were suspended in PUM buffer, and adherence to octane was carried out as described previously (9–14) in the presence of 100 μg of emulsan per ml (○) or in the absence of the biopolymer (●). Results are expressed as percentage of the initial absorbance at 400 nm of the aqueous phase (after mixing) as a function of hydrocarbon volume added.

medium and allowed to grow for 6 h before harvesting. *A. calcoaceticus* BD413 (4) was grown overnight in the same medium. *S. pyogenes* M-5 (H type 5) was grown at 37°C in brain heart infusion broth (Difco) under static conditions and harvested after 48 h.

Epithelial cells. Epithelial cells were obtained as described previously (11) by scraping the buccal mucosa of several healthy donors with wooden sticks. Samples were taken in the morning before the donors had put anything into their mouths. The cells were washed twice and suspended in phosphate-buffered saline to a concentration of 10^6 cells per ml. Viability of epithelial cells, as determined by trypan blue exclusion staining, was 40 to 60% in the presence or absence of emulsan.

Emulsan. Emulsan, the polyanionic emulsifying agent of *A. calcoaceticus* RAG-1, was purified from the cell-free supernatant fluid obtained from an ethanol-grown culture (8). After dialysis and lyophilization, the powder was washed in warm ethanol to remove colored impurities and then extracted into 80% ethanol; emulsan was then precipitated by the addition of a saturated solution of $Ca(NO_3)_2$ in 90% ethanol. The extraction of emulsan into 80% ethanol and precipitation with $Ca(NO_3)_2$ was repeated. The product was then dissolved in water, dialyzed extensively against distilled water, and lyophilized. Stock solutions of 2 mg of emulsan per ml were used routinely. The emulsan used in these studies had an emulsifying activity of 150 U/mg (8), an O-ester content of 0.5 μmol/mg, a reduced viscosity of 350 cm^3/g, and a residual protein content of less than 5%.

RESULTS

As previously demonstrated (10), *A. calcoaceticus* RAG-1 and BD413, as well as *S. pyogenes* M-5, adhered to octane (Fig. 1). Under the conditions employed, *A. calcoaceticus* RAG-1 cells were more adherent than *S. pyogenes*, which was slightly more adherent than *A. calcoaceticus* BD413. The adherence of all these strains to octane was inhibited by 100 μg of emulsan per ml. With the more hydrophobic RAG-1 cells, emulsan was more efficient at lower octane volumes, whereas with the other two strains, emulsan was effective over the entire range of octane concentrations examined. Higher concentrations of emulsion could not be used to prevent the bacteria from adhering to the hydrocarbon-water interface because of the formation of stable emulsions, which interfered with turbidity measurements. Similarly, the use of octane as a test hydrocarbon in these experiments prevented the formation of stable emulsions, as this hydrocarbon is poorly emulsified by emulsan (19).

The emulsan-mediated inhibition of RAG-1 adherence to octane was not due to a measurable change in cell viability nor to an irreversible binding of emulsan to the bacterial cell surface. Pretreatment of RAG-1 cells with 200 μg of emulsan per ml for 30 min had no effect on cell viability. Furthermore, when RAG-1 cells were harvested by centrifugation after emulsan treatment and then resuspended in PUM buffer (9), the cells adhered to hydrocarbons with the same efficiency as untreated controls.

As seen in Fig. 2, emulsan prevented adherence of RAG-1 to buccal epithelial cells. In the absence of emulsan, 70% of the epithelial cells contained more than 150 adherent RAG-1 cells, whereas in the presence of 100 μg of emulsan per ml, 67% of the epithelial cells had 50 or less adhering RAG-1 cells. The mean values of RAG-1 cells per epithelial cell were 221 and 40 for the control and emulsan-containing suspensions, respectively.

Emulsan was also effective in preventing adherence of *S. pyogenes* M-5 to epithelial cells (Fig. 3). In the absence of emulsan, 94% of the epithelial cells contained more than 20 adherent *S. pyogenes* cells, whereas in the presence of

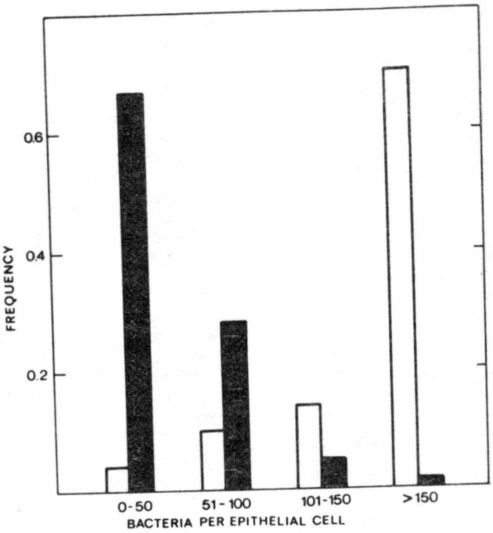

FIG. 2. Adherence of *A. calcoaceticus* RAG-1 to buccal epithelial cells. Equal volumes of washed *A. calcoaceticus* RAG-1 (1.0 × 10^9 cells per ml) and epithelial cells (1.1 × 10^6 cells per ml) were incubated together for 15 min in the presence of 100 μg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars), and adherence was measured after filtration, as described in the text. The data were pooled from three independent experiments in which a total of 600 epithelial cells were scored microscopically. The background values of adherent indigenous bacteria were subtracted in all cases.

200 μg of emulsan per ml, 61% of the epithelial cells had 20 or less adhering *S. pyogenes* cells. The mean values of *S. pyogenes* cells per epithelial cell were 57.2 and 20.7 for the control and emulsan-containing suspensions, respectively.

Emulsan, in addition to its ability to inhibit bacterial adherence to epithelial cells, was also found to be effective in desorbing previously bound RAG-1 cells (Fig. 4). Desorption of RAG-1 from the epithelial cells was dependent on the emulsan concentration. A maximum of 80% desorption was obtained at 200 μg of emulsan per ml.

While examining the effects of emulsan on adherence of *A. calcoaceticus* RAG-1 and *S. pyogenes* M-5 to epithelial cells, it became evident that a significant fraction of the bacterial population indigenous to the buccal epithelial cell surface was removed by the emulsan treatment. Thus, a study of emulsan-mediated desorption of the indigenous bacteria (with no bacteria added) was carried out (Fig. 5). The 500 control epithelial cells scored (no emulsan) showed a wide range in the number of bacteria per epithelial cell; the mean and median values of bacteria per epithelial cell were 61 and 39, respectively. Epithelial cells treated with 200 μg of emulsan per ml had mean and median values of bacteria per epithelial cell of 18 and 8, respectively. Thus, 70% of the bound indigenous bacteria were removed by emulsan.

Treatment of epithelial cells with 200 μg of emulsan per ml for 30 min had no significant effect on the viability of the epithelial cells as determined by trypan blue exclusion staining. However, when emulsan-treated epithelial cells were centrifuged, washed twice and resuspended in phosphate-buffered saline, adsorption of RAG-1 cells to them was inhibited by 82%, suggesting that the epithelial cell surface was either modified by emulsan or that emulsan bound to the epithelial cells with high affinity.

DISCUSSION

In a previous report, we observed that adherent *A. calcoaceticus* RAG-1 cells were desorbed from hydrocarbon droplets during growth on hexadecane (12). The possibility that this was due to the production of emulsan by the growing cells (9) led us to examine the efficacy of emulsan in inhibiting bacterial adherence to hydrocarbon and other hydrophobic surfaces.

The results presented here clearly demonstrate that emulsan inhibits RAG-1 adherence to hydrocarbon. Emulsan-mediated interference of

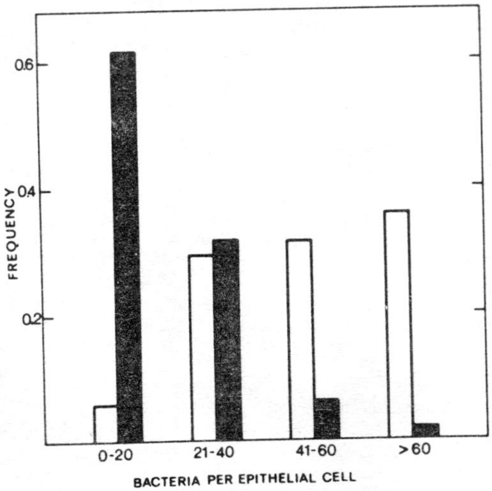

FIG. 3. Adherence of *S. pyogenes* M-5 to buccal epithelial cells. Equal volumes of washed *S. pyogenes* M-5 (1.0 × 10^9 cells per ml) and epithelial cells (1.1 × 10^6 cells per ml) were incubated together for 15 min in the presence of 200 μg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars). Nonadherent bacteria were removed by centrifugation at 100 × *g* for 5 min; epithelial cells were then suspended in phosphate-buffered saline, and adhering bacteria were measured as described in the text. Background values of adherent indigenous bacteria were subtracted.

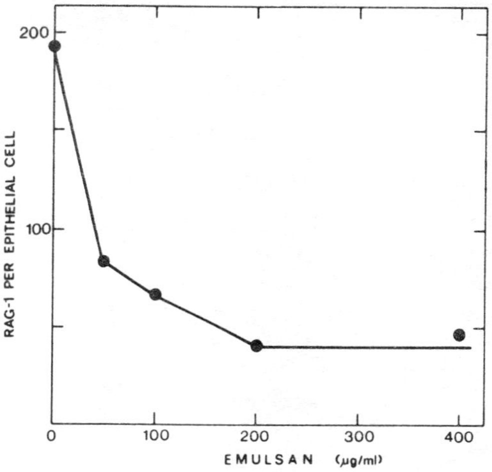

FIG. 4. Desorption of *A. calcoaceticus* RAG-1 from buccal epithelial cells by emulsan. After incubation of *A. calcoaceticus* RAG-1 and epithelial cells for 15 min as described in the legend to Fig. 2, the cell suspension was divided into five tubes, each containing different concentrations of emulsan. The mixtures were then incubated for an additional 15 min before filtration and determination of adhering bacteria. For each concentration of emulsan, the average number of bacteria per 100 epithelial cells was determined. Background values of adherent indigenous bacteria were subtracted.

adherence was neither limited to a particular bacterium nor specific for a particular hydrophobic surface. Adherence to hydrocarbons of another *A. calcoaceticus* strain, BD413, which produces a rhamnose-glucose exopolysaccharide (N. Kaplan and E. Rosenberg, submitted for publication) rather than emulsan, was also strongly inhibited by emulsan. Results similar to those obtained with *Acinetobacter* strains were also observed for *S. pyogenes* M-5; adherence of *S. pyogenes* to epithelial cells has been reported to be due to hydrophobic interactions resulting from the exposure of the lipid moiety of lipoteichoic acid (6).

In a previous study (11), correlations were found between the adherence of RAG-1 and *S. pyogenes* M-5 to hydrocarbons and adherence of these strains to buccal epithelial cells. It was thus of interest to determine whether emulsan would similarly inhibit adherence of these strains to buccal epithelial cells. Low emulsan concentrations (0.01 to 0.02%) proved highly effective in preventing adherence of both RAG-1 and *S. pyogenes* M-5 to buccal epithelial cells. In addition to its ability to prevent adherence, emulsan was able to remove 80% of RAG-1 cells which were previously bound to epithelial cells.

The amphipathic nature of the emulsan polymer (2, 20) enables it to coat hydrocarbon droplets with a thin, stable film which lowers the interfacial tension (8) and accounts for the excellent emulsifying properties of emulsan (8, 19). The data presented here demonstrate the ability of emulsan to act as a soluble antiadherence factor, inhibiting bacterial adherence to hydrocarbon and epithelial cells and removing bacteria bound to these surfaces. We propose that a common mechanism accounts for the potent emulsifying and antiadherence properties of emulsan: formation of a surface film which renders hydrophobic surfaces hydrophilic and prevents attachment of bacteria through hydrophobic interactions. It was previously suggested that hydrophobic interactions mediate adherence of RAG-1 and *S. pyogenes* M-5 to hydrocarbons and buccal epithelial cells (11). The ability of emulsan to inhibit adherence of these strains to both types of surfaces strengthens this contention. The reduced bacterial-binding ability of emulsan-pretreated epithelial cells suggests that emulsan either forms a film on epithelial cells in much the same manner as it coats hydrocarbon droplets or, alternatively, interferes with a bacterial receptor on the epithelial cell surface.

Of particular interest in this context is the effect of emulsan in removing adherent indigenous microbial flora from the buccal epithelial cell surface. Among 500 epithelial cells scored, 70% of the adherent autochthonous bacterial flora were removed by emulsan. These results suggest that hydrophobic interactions mediate not only the in vitro adherence of laboratory

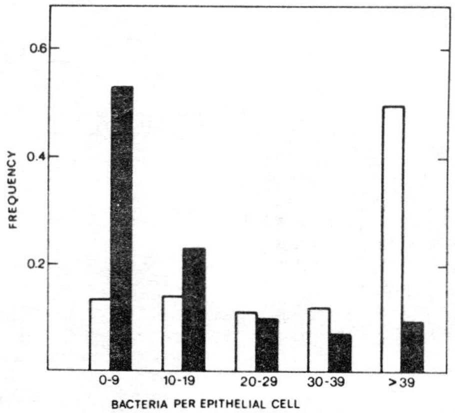

FIG. 5. Desorption of indigenous bacteria from buccal epithelial cells by emulsan. Washed human buccal epithelial cells were incubated for 15 min in the presence of 200 µg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars), and the remaining adherent bacteria were determined. The data were pooled from five independent experiments in which a total of 500 emulsan-treated epithelial cells and 500 control epithelial cells were examined.

strains to buccal epithelial cells, but may actually govern the adherence of the majority of the indigenous bacteria that normally populate this surface. Our preliminary finding that over 95% of the cultivable bacteria released from buccal epithelial cells partitioned to the hydrocarbon-water interface provides additional support for this concept. Thus, whereas surface hydrophobicity of pathogenic streptococcal strains such as M-5 may promote their adherence to and subsequent colonization of the buccal mucosa (1, 5, 6, 11, 16), similar mechanisms may enable the establishment of the natural flora on the epithelial cell surface.

The use of emulsan as an antiadherence agent has several advantages: (i) emulsan has been extensively purified and characterized, both physically and chemically (2, 8, 19, 20); (ii) it is stable over a wide pH range and retains activity after autoclaving; and (iii) as opposed to low-molecular-weight molecules, the polymeric nature of emulsan reduces the possibility of its penetration into the bacterial cell or mammalian tissue. Thus, it should be possible to use emulsan as a molecular probe in desorbing, isolating, and further characterizing bacteria which adhere with high affinity to hydrophobic surfaces.

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LITERATURE CITED

1. **Alkan, M., I. Ofek, and E. H. Beachey.** 1977. Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. Infect. Immun. **18:**555–557.
2. **Belsky, I., D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Acinetobacter calcoaceticus* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. **101:**175–178.
3. **Faris, A., T. Wadstrom, and J. H. Freer.** 1981. Hydrophobic adsorptive and hemagglutinating properties of *Escherichia coli* possessing colonization factor antigens (CFA/I or CFA/II), type 1 pili, or other pili. Curr. Microbiol. **5:**67–72.
4. **Juni, E., and A. Janick.** 1969. Transformation of *Acinetobacter calcoaceticus* (*Bacterium anitraum*). J. Bacteriol. **98:**281–288.
5. **Ofek, I., and E. H. Beachey.** 1980. General concepts and principles of bacterial adherence in animals and man, p. 1–31. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, Ltd., London.
6. **Ofek, I., W. A. Simpson, and E. H. Beachey.** 1982. Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. J. Bacteriol. **149:**426–433.
7. **Perers, L., L. Andaker, L. Edebo, O. Stendahl, and C. Tagesson.** 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Pathol. Microbiol. Scand. Sect. B **85:**308–316.
8. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37:**402–408.
9. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. Appl. Environ. Microbiol. **44:**929–937.
10. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:**29–33.
11. **Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg, and I. Ofek.** 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. **33:**29–33.
12. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane. J. Bacteriol. **148:**51–57.
13. **Rosenberg, M., E. Rosenberg, and D. Gutnick.** 1980. Bacterial adherence to hydrocarbons, p. 541–542. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.
14. **Rosenberg, M., S. Rottem, and E. Rosenberg.** 1982. Cell surface hydrophobicity of smooth and rough *Proteus mirabilis* strains as determined by adherence to hydrocarbons. FEMS Microbiol. Lett. **13:**167–169.
15. **Smyth, C. J., P. Jonsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjerten, and T. Wadstrom.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22:**462–472.
16. **Tylewska, S. K., S. Hjerten, and T. Wadstrom.** 1979. Contribution of M protein to the hydrophobic surface properties of *Streptococcus pyogenes*. FEMS Microbiol. Lett. **6:**249–253.
17. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32:**19–40.
18. **Wadstrom, T., C. J. Smyth, A. Faris, P. Jonsson, and J. H. Freer.** 1979. Hydrophobic adsorptive and haemagglutinating properties of enterotoxigenic *Escherichia coli* with different colonization factors: K88, K99 and colonization factor antigens and adherence factor, p. 29–55. *In* S. Acres (ed.), Proceedings of the Second International Symposium on Neonatal Diarrhea. Stuart Brandle Publishing Service, Edmonton, Canada.

18a. **Weiss, E., M. Rosenberg, H. Judes, and E. Rosenberg.** 1982. Cell surface hydrophobicity of adherent oral bacteria. Curr. Microbiol. **7:**125–128.

19. **Zosim, Z., D. Gutnick, and E. Rosenberg.** 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. Biotechnol. Bioeng. **24:**281–292.
20. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. **37:**414–420.

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Respiratory Control in *Micrococcus lysodeikticus*

Mel Rosenberg[1] and Ilan Friedberg[2]

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Abstract

The respiration rate of P_i-deprived cells of *Micrococcus lysodeikticus* is markedly increased by P_i, and returns to the original level following P_i consumption. The stimulation of the respiration was found to be specific for P_i and arsenate. Although succinate and valinomycin enchanced the respiration of both P_i-grown and P_i-deprived cells, only the latter could be further stimulated by P_i. The effect of P_i on the respiration rate was found to be concentration dependent. The control of respiration by P_i is due to its rapid uptake and its subsequent polymerization to polyphosphate via ATP. Both of these processes are coupled to proton influx into the cell, and thus stimulate the proton efflux and the respiration rate.

Key Words: Respiratory control; phosphate transport; polyphosphate; protonmotive force; *Micrococcus lysodeikticus*.

Introduction

Respiratory control has been defined as the stimulation of respiration rate by ADP and P_i, followed by return to the original rate on exhauston of either cofactor (Chance and Baltscheffsky, 1958; Racker, 1976). This phenomenon has been widely demonstrated in mitochondria, but not in bacterial cells, although the processes of respiration and ATP synthesis are similar in both systems (Chance and Williams, 1955a,b; Racker, 1976; Haddock and Jones, 1977; Harold, 1972). Growing *Escherichia coli* cells apparently respire at their maximal capacity, since the $\Delta\bar{\mu}_{H^+}$,[3] generated by respiration, is simultaneously depleted by energy-consuming processes, such as active transport and

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[1] Department of Human Microbiology and School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, 69978, Tel-Aviv, Israel.

[2] Department of Microbiology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, 69978, Tel-Aviv, Israel.

[3] $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons across the cell membrane; $\Delta\psi$, electrical potential across the cell membrane; RCR, respiratory control ratio = (respiration rate after addition)/(respiration rate before addition).

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ATP synthesis. Thus, only when cell growth is arrested, and the respiration rate reduced, can the respiratory control be detected (Burstein *et al.,* 1979; Tsuchiya and Rosen, 1980). Unlike mitochondria, the respiration of whole bacterial cells is not affected by exogenous ADP, which does not permeate the cell envelope (Lichtenstein *et al.,* 1960). Indeed, respiratory control in intact bacterial cells has been demonstrated only indirectly, using uncouplers of oxidative phosphorylation (Bovell *et al.,* 1963; Scholes and Mitchell, 1970; Burstein *et al.,* 1979; Tsuchiya and Rosen, 1980). The common denominator between the effects of uncouplers and ATP synthesis is the reduction of $\Delta\bar{\mu}_{H^+}$ and the subsequent increase in the rate of proton extrusion by the respiratory chain (Harold, 1972; Mitchell, 1973; Rosen and Kashket, 1978).

An alternative method for studying respiratory control, which circumvents the above-mentioned constraints, is to employ cell homogenates or isolated membrane particles in which a fraction of the membranal ATPase is exposed to the external medium. Yet, in cell-free systems, ADP, or ADP and P_i, exerted only a small stimulation of the respiration (Scocca and Pinchot, 1965, 1968; Revsin and Brodie, 1967; Jones and Hamilton, 1970; Eilerman *et al.,* 1970, 1971; Jones *et al.,* 1971a,b; Burstein *et al.,* 1979).

This paper describes, for the first time, the control of electron flow in the respiratory chain via ATP/ADP in intact bacterial cells in a direct manner. The direct demonstration of respiratory control was possible due to the unique phosphate metabolism of *Micrococcus lysodeikticus.* In this organism, both the P_i uptake system, driven by $\Delta\bar{\mu}_{H^+}$, and polyP synthesis via ATP, are markedly derepressed during P_i starvation (Friedberg and Avigad, 1968, 1970; Friedberg, 1977a,b; Alfasi *et al.,* 1979; Friedberg and Kaback, 1980). This correlation between the phosphate metabolism of *M. lysodeikticus* and its respiratory control is discussed.

Materials and Methods

Micrococcus lysodeikticus ATCC 4698 (Fleming) was grown on P_i-buffered defined medium, composed of salts, amino acids, and vitamins, at 30°C, with shaking, to late logarithmic phase. P_i-deprived cells were obtained by additional growth on P_i-less medium, in which P_i was replaced by triethanolamine-HCl buffer (Friedberg, 1977a).

The assays of P_i and arsenate uptake were carried out in P_i-grown and P_i-deprived cells. The cells were harvested, washed twice, and suspended in 50 mM triethanolamine-HCl buffer, pH 7.8, containing KCl and $MgSO_4$ as indicated in the experiments, at room temperature; the cell suspensions were maintained for about 30 min before the assay. The uptake measurements were initiated by addition of either $[^{32}P]$-P_i or $[^{74}As]$-arsenate (usually to a final concentration of 0.1 mM) to 1 ml of cell suspension (final volume) containing about 0.4 mg cell protein, in a 25-ml flat-bottom flask, to ensure adequate aeration. Samples of 200 μl were withdrawn at 30-sec time intervals, filtered through membrane filters (Schlicher and Schull, 0.45 μm porosity), and washed with 5 ml of the uptake medium. The radioactivity on the filters was measured with a Packard 3330 scintillation spectrometer, and the uptake rate estimated from the slope of radioactivity vs. time, which was linear for at least 2 min (Friedberg, 1977a).

Fractionation of P_i, phosphate esters, and polyphosphate (polyP) was carried out as previously described (Friedberg and Avigad, 1968) and assayed according to Avron (1960).

Oxygen consumption was measured with a Clark type electrode at 30°C (Beechy and Ribbons, 1972). A standard assay system contained 0.5–1.0 mg cell protein per milliliter, in 50 mM triethanolamine-HCl buffer, pH 7.8, 10 mM KCl, 5 mM $MgCl_2$, and other additions, as indicated in the experiments, in a final volume of 5 ml.

Protein was determined by the Biuret method using bovine serum albumin as a standard (Gornell *et al.*, 1949).

Results

Addition of P_i to P_i-deprived cells of *M. lysodeikticus* resulted in a fourfold increase in their respiration rate (Fig. 1 and Table I). When the external phosphate was consumed by the cells, the respiration rate returned to

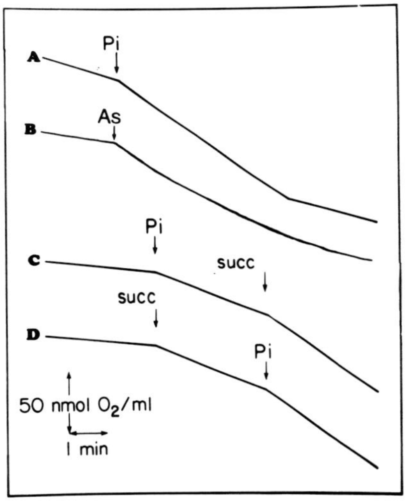

Fig. 1. Effects of P_i, arsenate, and succinate on oxygen consumption rate in P_i-deprived *M. lysodeikticus* cells. Cells were grown, starved for P_i, and prepared for oxygen consumption measurement as described in Materials and Methods. Reaction mixtures contained: (A) 1.5 mg protein per milliliter; P_i was added (arrow) to a final concentration of 80 μM. (B) 0.70 mg protein per milliliter; KH_2AsO_4 (As) was added (arrow) to a final concentration of 4.0 mM; (B, C) 0.38 and 0.33 mg protein per milliliter, respectively, and P_i and succinate (succ) were added (arrows) to final concentrations of 4.0 mM and 10.0 mM, respectively.

Table I. Stimulation of Respiration in P_i-Deprived and P_i-Grown Cells of *M. lysodeikticus*[a]

Additions	Respiratory control ratio	
	P_i-deprived cells	P_i-grown cells
Phosphate	3.9	1.0
Succinate	4.3	6.6
Phosphate + succinate	7.2	6.4
Succinate + phosphate	7.7	6.5
Arsenate	3.5	1.0
Valinomycin	3.9	3.3
Valinomycin + phosphate	5.3	3.3
Phosphate + valinomycin	5.0	3.1

[a] Cell growth, starvation for P_i, and measurements of respiration rates were carried out as described in Materials and Methods. Concentrations used: phosphate, 4 mM; succinate, 10 mM; arsenate, 4 mM; valinomycin, 4 μM. Respiratory control ratio = (respiration rate with addition)/(respiration rate without addition). Second addition was introduced 3 min after the first addition. The endogenous respiration rates (no addition) were 7.6 (±1.8) and 7.0 (±1.2) nmol O_2 min^{-1} mg^{-1} of cell protein for P_i-deprived and P_i-grown cells, respectively.

its original value (Fig. 1A). Arsenate, a nonmetabolized analogue of P_i [the uptake of which is mediated by the P_i transport system (Friedberg, 1977a; Alfasi *et al.*, 1979)], exerted a similar increase in the respiration rate (Table I). However, after 3 min the respiration rate gradually decrease, presumably due to the cytotoxicity of arsenate (Fig. 1B). Arsenate inhibits phosphate-dependent synthesis processes, such as polyP synthesis, even in the presence of P_i (Z. Eshhar and I. Friedberg, unpublished data). The stimulation of the respiration rate was specific for P_i and arsenate, as other salts, such as KCl, NaCl, $MgCl_2$, and $MgSO_4$ had no such effect. P_i and arsenate did not affect the respiration rate in P_i-grown cells (Table I).

Addition of succinate, either to P_i-grown or to P_i-deprived cell suspensions, in buffered salt solution, resulted in an increase in the respiration rate (Fig. 1 and Table I). The stimulation of the respiration in P_i-grown cells was about 50% higher than that obtained with P_i-deprived cells. However, only in P_i-deprived cells was an additional stimulation exerted by P_i. The effects of P_i and succinate on the respiration rate in P_i-deprived cells appeared to be mutually independent since an equivalent increase in the RCR exerted by P_i was observed in the presence (increase of 3.8) or absence (increase of 3.9) of succinate. Similarly, the increase of RCR due to succinate addition was approximately the same before (increase of 4.3) or after (increase of 3.3) P_i addition (Fig. 1 and Table I).

Valinomycin, an ionophore for K^+, which effects $\Delta\psi$, exerted an increase of 3- to 4-fold in RCR, both in P_i-grown and P_i-derived cells. However, an additional increase due to P_i addition was obtained only in P_i-deprived cells (Table I).

The magnitude of the stimulation of respiration upon P_i addition to

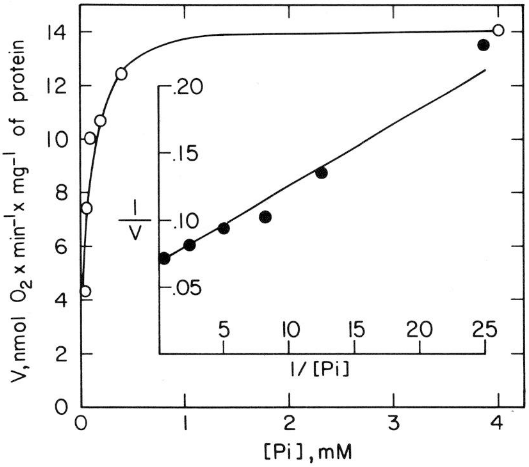

Fig. 2. Increase in the initial rate of respiration (V) as a function of P_i concentration [P_i], in P_i-deprived cells of *M. lysodeikticus*. Growth, starvation for P_i, and measurements of oxygen consumption are as described in Materials and Methods. Inset: a double reciprocal plot (Lineweaver–Burk) of $1/V$ vs. $1/[P_i]$.

P_i-deprived cells was concentration dependent; typical saturation kinetics were observed (Fig. 2). The P_i concentration required for half maximal effect on the respiration rate was estimated to be 85 μM (Fig. 2).

The P:O ratio in P_i-deprived *M. lysodeikticus* cells could be estimated from simultaneous measurements of oxygen consumption and incorporation of P_i into polyP (via ATP). In a typical experiment, a cell suspension of P_i-deprived cells in buffered salt solution was prepared as described in Materials and Methods, containing 1 mg of cell protein per milliliter. [^{32}P]-P_i was added to a final concentration of 0.1 mM. During the first 2 min after P_i addition, 65.6 nmol P_i per milliliter underwent polymerization into trichloroacetic acid-insoluble polymers [mainly polyP (Friedberg and Avigad, 1970)], and 0.9 nmol phosphate per milliliter were found to be soluble phosphate esters (Avron, 1960). The increase in oxygen consumption due to P_i addition was 66 ng-atom O per milliliter. Thus, the P:O ratio was calculated to be 1.0 in P_i-deprived *M. lysodeikticus* cells.

Discussion

The effect of P_i on the respiration rate of *M. lysodeikticus* reflects a mechanism of respiratory control. The direct demonstration of respiratory control in intact bacterial cells was effected due to the rapid P_i uptake and its subsequent polymerization to polyP, via ATP, in P_i-deprived cells of *M. lysodeikticus* (Kornberg *et al.*, 1956; Friedberg and Avigad, 1968, 1970). The postulated effects of P_i uptake are summarized in Fig. 3. The active transport of P_i, as well as ATP synthesis mediated by membranal ATPase, was shown to be coupled to proton influx (Friedberg, 1977a,b; Alfasi *et al.*, 1979; Friedberg and Kaback, 1980; Huberman and Salton, 1979). ATP is continuously synthesized, as both P_i and ADP are readily available, by the processes of active transport and polyP synthesis, respectively. The continuous proton influx releases the back pressure on the respiratory chain, resulting in an increase in proton efflux and oxygen consumption (Scholes and Mitchell, 1970). Upon exhauston of external P_i, both P_i uptake and polyP synthesis are arrested, and the respiration rate decreases to its original value.

The increase in oxygen consumption elicited by valinomycin is in agreement with the hypothesis that the stimulation of respiration observed here is due to reduction of $\Delta\bar{\mu}_{H^+}$. Valinomycin was shown to decrease $\Delta\bar{\mu}_{H^+}$ in *M. lysodeikticus* (Friedberg, 1977b; Friedberg and Kaback, 1980). Stimulation of respiration elicited by ionophores was reported in additional bacterial strains (Bovell *et al.*, 1963; Scholes and Mitchell, 1970; Burstein *et al.*, 1979;

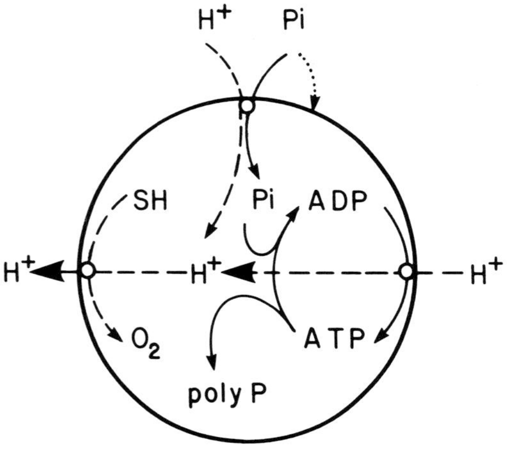

Fig. 3. Schematic presentation of the release of respiratory control by P_i in P_i-deprived *M. lysodeikticus* cells. P_i transport is coupled to proton influx. P_i polymerization to polyP via ATP results in proton influx by the membranal ATPase. Both processes decrease $\Delta\bar{\mu}_{H^+}$ and thus increase proton extrusion by the respiratory chain and oxygen consumption. P_i also affects the membrane externally. Symbols: SH, oxidizable substrate; polyP, polyphosphate.

Tsuchiya and Rosen, 1980). Valinomycin elicited an increase in RCR both in P_i-grown and P_i-deprived cells. However, only in the latter was further stimulation obtained upon P_i addition, indicating an additional dissipation of $\Delta\bar{\mu}_{H^+}$ due to P_i uptake and its metabolism. Under the conditions employed, especially the low external concentration of KCl (10 mM), valinomycin induces K^+ efflux down its concentration gradient, and generation of a diffusion potential across the cell membrane, negative inside. The diffusion potential partially counteracted the dissipation of $\Delta\bar{\mu}_{H^+}$. Thus, the overall potential was indeed reduced, but not abolished (Harold *et al.*, 1975). Then P_i uptake and its subsequent metabolism in P_i-deprived cells elicited an additional dissipation of $\Delta\bar{\mu}_{H^+}$, which was expressed as an additional increase in RCR.

The differences between the P_i concentration required for half-maximal effect on the respiration rate (85 μM) and the K_m for P_i transport (4.3 μM; Friedberg, 1977a) are explained by an additional, exogenous effect of P_i on the integrity of the cell membrane. Thus, proline uptake in membrane vesicles of *M. lysodeikticus* was found to be more efficient when the vesicles were isolated in buffers containing P_i, as compared to P_i-less buffers (I. Friedberg and H.R. Kaback, unpublished observation). In addition, P_i increased the rate of NADH oxidation in particles of *M. lysodeikticus,* and this increase was not affected by ADP (Ishikawa and Lehninger, 1962).

Since most of the P_i taken up to P_i-starved cells is rapidly polymerized to polyP via ATP, we estimated the P:O ratio by simultaneous measurements of polyP synthesis and oxygen consumption. The method is fast and reproducible. and the value of the P:O ratio in intact, P_i-deprived cells of *M. lysodeikticus* was found to be 1. A similar value was reported, employing membrane fragments of *M. lysodeikticus* (Ishikawa and Lehninger, 1962).

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References

Alfasi, H., Friedberg, D., and Friedberg, I. (1979). *J. Bacteriol.* **137,** 69–72.
Avron, M. (1960). *Biochim. Biophys. Acta* **40,** 257–272.
Beechey, R. B., and Ribbons, W. D. (1972). In *Methods in Microbiology* (Norris, J. R., and Ribbons, D. W., eds.), Vol. 6, Academic Press, New York, pp. 25–54.
Bovell, C. R., Packer, L., and Helgerson, R. (1963). *Biochim. Biophys. Acta* **75,** 257–266.

Burstein, C., Tiankova, L., and Kepes, A. (1979). *Eur. J. Biochem.* **94,** 387–392.
Chance, B., and Williams, G. R. (1955a). *J. Biol. Chem.* **217,** 383–393.
Chance, B., and Williams, G. R. (1955b). *Adv. Enzymol.* **17,** 65–134.
Chance, B., and Baltscheffsky, H. (1958). *Biochem. J.* **68,** 283–297.
Eilerman, L. J. M., Pandit-Hovenkamp, H. G., and Kolk, A. H. J. (1970). *Biochim. Biophys. Acta* **197,** 25–30.
Eilerman, L. J. M., Pandit-Hovenkamp, H. G., Van der Meer-Van Buren, M., Kolk, A. H. J., and Feenstra, M. (1971). *Biochim. Biophys. Acta* **245,** 305–312.
Friedberg, I. (1977a). *Biochim. Biophys. Acta* **466,** 451–460.
Friedberg, I. (1977b). *FEBS Lett.* **81,** 264–266.
Friedberg, I., and Avigad, G. (1968). *J. Bacteriol.* **96,** 544–553.
Friedberg, I., and Avigad, G. (1970). *Israel J. Med. Sci.* **6,** 511–518.
Friedberg, I., and Kaback, H. R. (1980). *J. Bacteriol.* **142,** 651–658.
Gornell, A. G., Bardawill, C. J., and David, N. M. (1949). *J. Biol. Chem.* **177,** 751–766.
Haddock, B. A., and Jones, C. W. (1977). *Bacteriol. Rev.* **41,** 47–99.
Harold, F. M. (1972). *Bacteriol. Rev.* **36,** 172–230.
Harold, F. M., Altendorf, K. H., and Hirata, H. (1975). *Ann. N.Y. Acad. Sci.* **235,** 149–160.
Huberman, M., and Salton, M. R. J. (1979). *Biochim. Biophys. Acta* **547,** 230–240.
Ishikawa, S., and Lehninger, A. L. (1962). *J. Biol. Chem.* **237,** 2401–2408.
Jones, P., and Hamilton, W. A. (1970). *FEBS Lett.* **10,** 246–248.
Jones, C. W., Erickson, S. K., and Ackrell, B. A. C. (1971a). *FEBS Lett.* **13,** 33–35.
Jones, C. W., Ackrell, B. A. C., and Erickson, S. K. (1971b). *Biochim. Biophys. Acta* **245,** 54–62.
Kornberg, A., Kornberg, S. R., and Simms, E. S. (1956). *Biochim. Biophys. Acta* **20,** 215–227.
Lichtenstein, J., Barner, H. D., and Cohn, S. S. (1960). *J. Biol. Chem.* **235,** 457–465.
Mitchell, P. (1973). *J. Bioenerg.* **4,** 63–91.
Racker, E. (1976). *A New Look at Mechanisms in Bioenergetics,* Academic Press, New York.
Revsin, B., and Brodie, A. F. (1967). *Biochem. Biophys. Res. Commun.* **28,** 635–640.
Rosen, B. P., and Kashket, E. R. (1978). In *Bacterial Transport* (Rosen, B. P., ed.), Marcel Dekker, New York, pp. 559–620.
Scholes, P., and Mitchell, P. (1970). *J. Bioenerg.* **1,** 61–72.
Scocca, J. J., and Pinchot, G. B. (1965). *Fed. Proc.* **24,** 544.
Scocca, J. J., and Pinchot, G. B. (1968). *Arch. Biochem. Biophys.* **124,** 206–217.
Tsuchiya, T., and Rosen, B. P. (1980). *FEBS Lett.* **120,** 128–130.

DENTAL MEDICINE

רפואת הש״נים
JAN 1985

Adherence of a non-oral hydrophobic bacterium to the human tooth surface

Ervin Weiss, D.M.D., Herbert Judes, B.D.S. (Rand),* Mel Rosenberg, Ph.D.,***

In many bacterial diseases, the adherence of microorganisms to the surfaces of host cells is the initial step in the infectious process. For Example, streptococcal induced pharyngitis (1), endocarditis (2) and gonorrhea (3) appear to require attachment of the bacteria to the host tissue prior to the development of pathogenesis. Similarly, in the oral cavity, bacterial adherence to and subsequent accumulation on the tooth surface are prerequisites for the initiation of dental disease (4-6).

Much research has focused on elucidation of the mechanisms which mediate bacterial attachment to the tooth surface and elsewhere in the oral cavity (5,6). An increasing number of recent investigations (7-18) have suggested that hyprophobic interactions play an important role in mediating a wide variety of adherence phenomena, including attachment of bacteria to the acquired pellicle covering the tooth surface (7-14) and to buccal epithelial cells (17,18). There is, however, some disagreement as to the extent played by hydrophobic interactions in mediating bacterial adherence within the oral cavity. Is pronounced cell surface hydrophobicity sufficient per se to enable bacterial attachment to oral surfaces (7-9), or do hydrophobic interactions complement more specific adherence mechanism (10-14), such as lectin-like interactions? One way to resolve this question is to compare adherence of hydrophobic strains to tooth surface models with that of corresponding nonhydrophobic mutants (11). If oral strains are employed, however, it is possible that loss of hydrophobic surface components is concomitant with disappearance of other cell surface factors which contribute to adherence via alternative specific mechanisms.

In previous reports (17,18) non-specific hydrophobic interactions were shown to mediate bacterial adherence to buccal epithelial cells. These studies compared adherence of the non-oral, hydrophobic marine bacterium Acinetobacter calcoaceticus RAG-1 with that of its nonhydrophobic mutant, MR-481. In the present report, we have used a similar approach to study bacterial attachment to the tooth surface. Results obtained both in vitro and in situ indicate that loss of cell surface hydrophobicity correlates with poor adherence to the tooth surface, and suggest that hydrophobic interactions per se can mediate attachment of a "foreign" bacterium to the tooth surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Acinetobacter calcoaceticus RAG-1 (ATCC 31012) and its nonhydrophobic mutant, MR-481, were used in this study. Isolation of RAG-1 and MR-481 have been previously described and their growth and adherence properties compared (16,18). Cells were maintained on nutrient agar at 4°C and were subcultured every two months. RAG-1 and MR-481 cells were grown with shaking to stationary phase (18 h) on brain heart infusion broth (Difco), washed twice and suspended in either PUM buffer (22.2 g$K_2HPO_4.3H_20$; 7.26 KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4.7H_20$, and distilled water to 1000 ml, pH 7.1), or PBS (phosphate-buffered saline) as indicated.

Adherence of RAG-1 and MR-481 to the tooth surface (in vitro). RAG-1 and MR-481 cells were washed twice and suspended in PUM buffer to an initial concentration of ca. 10^9 cell/ml. RAG-1 and MR-481 cells were mixed so as to obtain a majority of MR-481 cells. Two teeth, freshly extracted from the same individual, were rinsed in PUM buffer. The crown of one tooth was immersed in the mixed bacterial suspension for 10 min. Te crown of the second tooth was incubated in PUM alone. Following incubation, the teeth were rinsed three times with PUM buffer. Each tooth was suspended in 10 ml PUM and exposed to two 30 second periods of sonic oscillation in an ultrasonic dental scaler (Beta Ltd., Beersheba, Israel) to remove bound cells. Appropriate serial dilutions were made on brain heart infusion agar plates of the initial cell mixture and of the cells released from the tooth surfaces. Plates were incubated for 24 h at 30°C under aerobic conditions. Colonies were counted, and compared for their ability to replicate onto polystyrene disks (16).

Adherence of RAG-1 and MR-481 to the tooth surface (in situ). RAG-1 and MR-481 were washed twice and suspended in PBS to a turbidity of 1000 Klett units (Klett-

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* Section of Operative Dentistry
** Dept. of Human Microbology and School of Dental Medicine

Summerson colorimeter fitted with a green filter), corresponding to ca 10^{10} cells/ml. Seven µl drops of each bacterial suspension were directly applied to the labial surfaces of adjacent upper central incisors protruding through a rubber dam, of a healthy, low caries volunteer. Following 5 min incubation of the cell suspensions on the teeth in situ, excess cell suspension was removed by suction; the tooth surfaces were extensively rinsed with tap water, then fixed with ethanol and stained with Gentian Violet (16).

Adherence experiments were performed at room temperature.

RESULTS

Adherence of RAG-1 and MR-481 to the human tooth surface was tested by quantitative and qualitative experiments. In the former, the crown of a freshly extracted human tooth was incubated in a mixed suspension of RAG-1 and MR-481 cells (Table 1). In the original cell mixture, the ratio of RAG-1:MR-481 cells was 0.36:1.0. Among the bacterial cells released from the tooth surface, the ratio of hydrophobic RAG-1 to nonhydrophobic MR'481 cells was 3.0:1.0, representing an 8.3 fold enrichment in the adherence of RAG-1 over MR-481 cells, as compared to their proportions in the initial mixture.

In a qualitative experiment aimed at comparing the ability of RAG-1 and MR-481 to adhere to the tooth surface in situ drops of bacterial suspensions of each were applied to adjacent teeth. Following washing and staining, it was evident that an aherent film remained on the tooth surface in the place of the drop of RAG-1 cells, whereas MR-481 cells were completely washed from the tooth surface (Fig. 1). Similar results (not shown) were obtained using freshly extracted human teeth.

DISCUSSION

Acinetobacter calcoaceticus RAG-1 is a Gram-negative bacterium which has been shown to adhere to a variety of surfaces, including hydrocarbons (15), polystyrene (16), and human buccal epithelial cells (18). Nonhydrophobic mutant MR-481 was shown to the deficient in its ability to adhere to all the above substrata. In the present study, we have extended this list to include adherence in situ and in vitro to the human tooth surface.

Adherence to the tooth surface could be demonstrated both by qualitative staining of the tooth surface in situ, as well as by a quantitative comparison of adherence to extracted teeth in vitro. In the former, RAG-1 cells were clearly adherent, whereas MR-481 were not. In the second experiment an 8.3 fold enrichment of RAG-1:MR-481 cells released from the tooth surface was recorded. This value is similar to that obtained in a comparison of adherence of these strains to epithelial cells (18). Since only cells which were released from the tooth surface following sonic oscillation were counted, a proportion of RAG-1 cells which may have remained bound were not considered. Thus the value obtained here may be considered to be minimal. The proportion of normal flora released was judged to be negligible, based on the results obtained with a control tooth incubated in buffer alone (Table 1).

Various lines of experimentation have recently shown that (i) a high proportion of oral strains exhibit high cell surface hydrophobicity (7,8,10-14); (ii) a general correlation exists between cell surface hydrohobicity and adherence to the tooth surface model, saliva-coated hydroxyapatite (10,11,13), and (iii) the tooth surface in situ is covered by a pellicle of low surface energy (19,20). Several investigators have suggested that bacterial adherence to the tooth surface is mediated by a combination of hydrophobic interactions and specific lectin-like mechanisms (10,13). The results presented here support the alternative contention that 'non-specific' hydrophobic interactions per se are sufficient to enable bacterial adherence to the tooth surface.

Table 1: **Adherence of RAG-1 and MR-481 to the crown of an extracted human tooth.**

Suspension	No. of colonies examined[1]	No. of Rag-1 colonies[2]	Ratio of RAG-1:MR-481
Incubation mixture	222	59	0.36:1
Released	197	148	3.0:1

[1] The crown of a freshly extracted human tooth was immersed into an incubation mixture consisting of RAG-1 and MR-481 cells which were previously washed and suspended in PUM buffer. Following 10 min incubation, the tooth was rinsed, then exposed to sonic oscillation in order to remove adherent cells. The crown of a second tooth, extracted from the same patient at the same time was immersed into PUM buffer alone, washed and exposed to sonic oscillation. Appropriate dilutions of the original cell mixture, and the mixtures released following sonic oscillation were plated onto brain heart infusion agar. The total cfu/ml in the original mixture was 1.5×10^{9}; a total of 1.6×10^{6} cfu were recovered from the tooth incubated in the cell mixture. The colony forming units recovered from the control tooth, incubated in PUM alone, were less than 1% of those recovered from the tooth incubated in the cell mixture.

[2] RAG-1 and MR-481 colonies were distinguished by the ability of the former to adhere to a polystyrene replica (16).

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REFERENCES

1 Alkan, M., Ofek, I., and Beachey, E.H.: Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. Infect. Immun. 18:555-557, 1977.

2 Gould, K., Ramirez-Ronda, C.H., Holmes, R.K., and Sanford, J.P.: Adherence of bacteria to heart valves in vitro. J. Clin. Invest. 56:1364-1370, 1975.

3 Ward, M.E., and Watt, P.J.: Adherence of Neisseria to urethral mucosal cells: an electron microscopic study of human gonorrhea. J. Infect. Dis. 126:601-605, 1972.

4 Loe, H., Theilade, E., and Jensen, S.B.: Experimental gingivitis in man. J. Periodontol. 36:177-187, 1965.

5. Gibbons, R.J.: Attachment of oral streptococci to mucosal surfaces. In (D. Schlessinger, Ed.), American Society for Microbiology, Washington, D.C. Microbiology - 1975, pp. 127-131, 1975.

6. Hamada, S., and Slade, H.D.: Biology, immunology and cariogenicity of Streptococcus mutans. Microbiol. Rev. 44:331-384, 1980.

7 Weiss, E., Rosenberg, M., Judes, H., and Rosenberg, E.: (1982) Cell surface hydrophobicity of adherent oral bacteria. Current Microbiology 7:125:128, 1982.

8 Rosenberg, M., Judes, H., and Weiss, E. (1983): Cell surface hydrophobicity of dental placque microorganisms in situ. Infect. Immun. "in press".

9 Rosenberg, M., Rosenberg, E., Judes, H., and Weiss, E. (1983): Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. FEMS Microbiol. Lett. "in press".

10. Gibbons, R.J., and Etherden, I. (1983): Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicies. Infect. Immun. "in press".

11. Gibbons, R.J., Etherden, I., and Skobe, Z.: Association of fimbrie with the hydrophobicity of Streptococcus sanguis FC-1 and adherence to salivary pellicles. Infect. Immun 41:414-417.

12. Doyle, R.J., Nesbitt, W.E., and Taylor, K.G.: On the mechanism of adherence of Streptococcus sanguis to hydroxylapatite. FEMS Microbiol. Lett. 15:1-5, 1982.

13. Nesbitt, W.E., Doyle, R.J., and Taylor, K.G.: Hydrophobic interactions and the adherence of Streptococcus sanguis to hydroxylapatite. Infect. Immun. 38:637-644, 1982.

14. Olsson, J., and Westergren, G.: Hydrophobic surface properties of oral streptococci. FEMS Microbiol. Lett 15:319-323, 1982.

15. Rosenberg, M., Gutnick, D., and Rosenberg, E.: Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33, 1980.

16. Rosenberg, M.: Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. 42:375-377, 1981.

17. Rosenberg, E., Gottlieb, A., and Rosenberg, M.: 1983. Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. Infect. Immun. 39:1024-1028, 1983.

18. Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E., and Ofek, I.: Adherence of Acinetobacter calcoaceticus RAG-1 to human epithelial cells and to hexadecane. Infect. Immun. 33:29-33, 1981.

19. Glantz, P.O.: On wettability and adhesiveness. A study of enamel, dentine, some restorative materials, and dental plaque. Odontologisk Revy 20 (Suppl. 17): 1-132, 1969.

20. Glantz, P.O.: 1977. Adhesion to teeth. International Dental Journal 27:324-332, 1977.

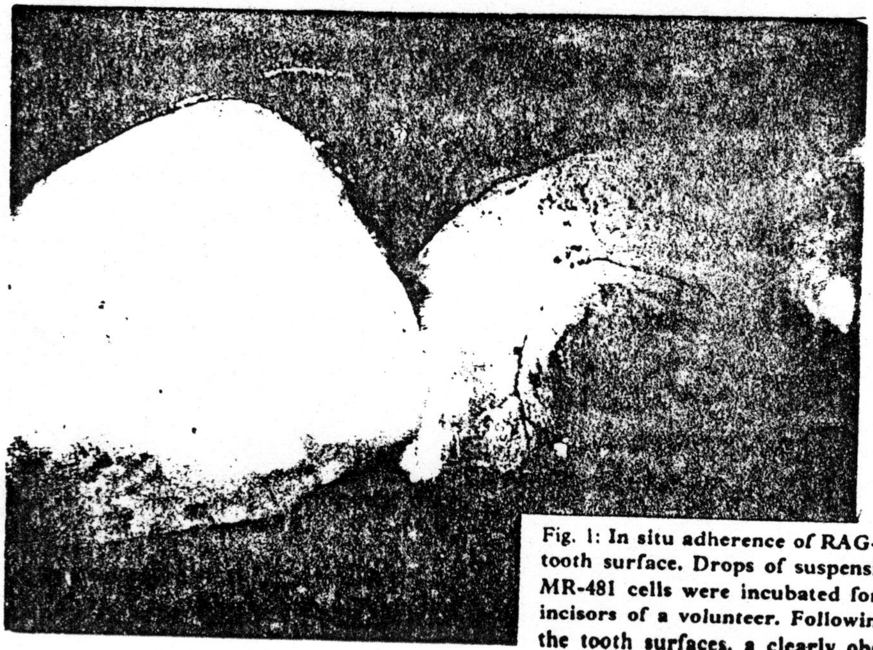

Fig. 1: In situ adherence of RAG-1 vs. MR-481 to the human tooth surface. Drops of suspensions of washed RAG-1 and MR-481 cells were incubated for 5 min on adjacent upper incisors of a volunteer. Following washing and staining of the tooth surfaces, a clearly observable layer of adherent RAG-1 cells remained on the tooth (right) to which the RAG-1 droplet was applied. In contrast, MR-481 cells were washed from the surface of the tooth on left.

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Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity

(Hydrophobicity; adherence; hydrocarbons; bacteria; cell-surface; method)

Mel Rosenberg

School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel

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1. SUMMARY

Bacterial adherence to hydrocarbons (BATH) is a simple and rapid technique for determining cell-surface hydrophobicity. During recent years, this method has found application in the study of the surface characteristics of a wide variety of bacteria and bacterial mixtures. Correlations have been found between the adherence of bacteria to hydrocarbons and their attachment to other surfaces, including non-wettable plastics, epithelial cells, and teeth. A slight modification of the assay enables the isolation of nonhydrophobic mutants. The present publication briefly describes the technique and its modifications, summarizes results obtained using this method, and suggests several directions for further investigation.

2. INTRODUCTION

Bacterial adherence to oils was observed and documented in the pioneering studies of Mudd and Mudd [1,2] some 60 years ago, and more recently, in reports by K.C. Marshall and coworkers, on the polar adhesion of *Hyphomicrobium, Flexibacter* and *Rhizobium* strains at the oil : water interface [3,4]. Despite these and other published data, researchers in the field of petroleum microbiology have often considered adherence to oil to be a specific property of oil-degrading microorganisms [5–7].

Our interest in the possible role of adherence in growth of the marine bacterium, *Acinetobacter calcoaceticus* RAG-1, on hexadecane led us to develop a simple quantitative assay to test this question [8]. Initial results showed that (i) although RAG-1 adheres avidly to hydrocarbons which it metabolizes (e.g. hexadecane), it adheres just as well to other hydrocarbons which it cannot degrade [8], and (ii) certain other bacteria, such as *Serratia marcescens,* which are known to possess hydrophobic surface properties [9–14], adhere avidly to hydrocarbons, despite their inability to degrade them [8]. These results led us to suggest that bacterial adherence to hydrocarbons (BATH) could serve as a general, quantitative assay for measuring cell-surface hydrophobicity [8,12,13]. Since then, BATH has been employed in a variety of investigations concerned with the microbial cell surface.

The purpose of the present manuscript is (i) to describe aspects pertaining to the technique itself; (ii) to present some of the results obtained employing BATH, and (iii) to suggest several avenues for further investigation.

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BATH—METHODOLOGICAL ASPECTS

Details of the basic technique for measuring bacterial adherence to hydrocarbons have been described [8,12,13], and can be summarized as follows: a turbid, aqueous suspension of washed microbial cells is mixed by vortexing in the presence of a test liquid hydrocarbon under controlled conditions. Following mixing, the two phases are allowed to separate. In the case of adherence, cells from the bulk aqueous phase become bound to hydrocarbon droplets and rise with them following the mixing to form an upper 'cream' consisting of cell-coated oil droplets. When nonadherent bacteria are tested, the phases separate following the mixing procedure, with the cells remaining in the bulk aqueous suspension. The percentage of adherent cells can easily be ascertained by the decrease in absorbance of the lower aqueous phase following the assay, as compared to the absorbance of the original bacterial suspension. Alternatively, the cells may be radiolabelled, and the radioactivity remaining in the aqueous phase measured [11]. It is also possible to perform viable counts prior to and following the mixing procedure, provided that proper controls are undertaken (E. Rosenberg, personal communication).

Various details which were originally employed in the paper describing the assay have since been modified and amended in various investigations. Parameters which have been altered include the suspending buffer [14,17–23], the volumes and ratios of the phases employed [15–21,23,24], the test tubes used [15,19,20,23,24], duration of mixing [15,16,18–21,23,24], and the test hydrocarbons employed [20]. Results obtained in this and other laboratories have shown that several aspects of the assay appear to be of major importance:

(i) The aqueous bacterial suspension must be free of surfactants, which inhibit the assay [8,21–23,25]. Similarly, the test tubes must be uniformly clean, preferably acid-washed [8].

(ii) Both the lower aqueous phase and the upper 'cream' should be examined microscopically to ensure that the cells are indeed at the hydrocarbon : water interface, and that the decrease in turbidity is not due to some other factor, such as clumping or lysis.

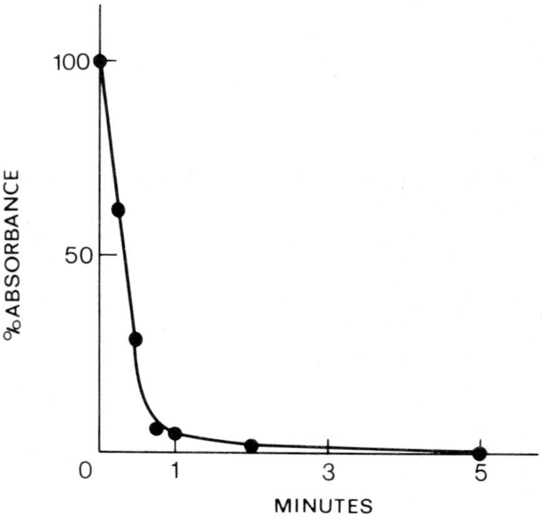

Fig. 1. Adherence to hexadecane as a function of duration of the mixing procedure. *Acetinetobacter calcoaceticus* RAG-1 cells were grown to early stationary phase on nutrient broth, washed and suspended in PUM buffer [8] to an initial absorbance at 400 nm of 1.48. To 1.2 ml aqueous cell suspension, 0.2 ml hexadecane were added and the mixtures vortexed for different lengths of time. All other assay conditions were as described in [8].

(iii) The assay can be 'overloaded' if the initial bacterial suspension is too concentrated. Thus, various bacterial concentrations should be assayed at the outset of any investigation.

(iv) Insufficient mixing may not allow for maximal bacterial adherence. This can be easily ascertained by increasing the duration of the vortexing procedure (Fig. 1 [13]). A flat-top mixer (such as the Multitube vortexer, SMI, or the Thermolyne maxi mix, Sybron) is preferable to single-tube vortexers, in that a number of tubes can be mixed simultaneously.

(v) The ionic strength and composition of the buffer employed may alter results. Several investigators have reported decreased adherence as a function of lowering the ionic strength of the suspending buffer [19,20,23].

(vi) Since the adherence of a given strain may largely depend on the growth conditions employed [8,11,13,17,21,23,27], adherence should be checked at various phases of growth, at different growth temperatures, and following growth on different media before proclaiming a given strain to be 'nonadherent'.

3.1. Mutant isolation and cell separations

Under optimal conditions, certain bacterial strains have been found to adhere to hydrocarbons to the extent of approx. 95% or more. In such cases, a slight modification of the adherence assay yields an enrichment technique for the isolation of nonhydrophobic mutants [26]. In this procedure, washed wild-type cells are vortexed in the presence of hexadecane or octane under sterile conditions. The lower aqueous phase is then removed and again extracted with hydrocarbon until the cells remaining in the aqueous phase are too few to be seen under microscopic examination. The lower aqueous phase is then inoculated into growth medium. Following growth, the cells are harvested, washed, and again mixed with the hydrocarbon as before. Following several extractions, the clear lower aqueous phase is once again inoculated into growth medium. Following several growth and extraction cycles, it may be observed that the test hydrocarbon is no longer able to extract the vast majority of cells. At this stage the lower aqueous phase is plated out and individual clones are assayed for BATH. Thus far, this technique has enabled isolation of nonhydrophobic mutants of *Acinetobacter calcoaceticus* [26], *Streptococcus sanguis* [16], and *Serratia marcescens* (M. Rosenberg, unpublished data). It would be of interest to determine whether other adherent strains yield nonhydrophobic mutants following this procedure.

BATH may also be employed to separate hydrophobic and hydrophilic cell mixtures (e.g., hydrophobic spores from vegetative cells), as shown in Fig. 2.

4. RESULTS OBTAINED USING BATH

A summary of some recent investigations which employed BATH, and the main results obtained, is presented in Table 1. Several lines of research which use this technique are currently in progress. These include studies on gliding motility in *Cytophaga johnsonae* (R. Wolkin, personal communication), swarming in *Proteus mirabilis* (Gail Johnston, personal communication), the relationship between phase variation in *Bordetella pertussis* and cell-surface hydrophobicity (F. Fish and S. Goldman, personal communication), the effect of specific growth rate on adherence and hydrophobicity of oral *Actinomyces* (R.P. Ellen and I. Hamilton, unpublished results), and the possible

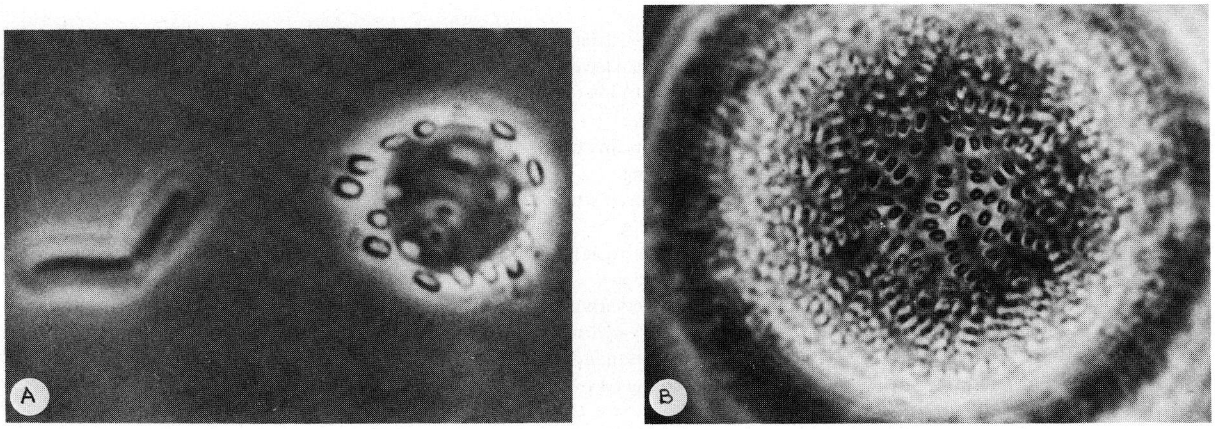

Fig. 2. Adherence of *Bacillus cereus* endospores to hexadecane. When a mixture of vegetative and endospore cells of *B. cereus*, scraped from a nutrient agar plate following 72 h incubation and suspended in PUM buffer [8], was mixed in the presence of hexadecane, spore cells adhered to the hydrocarbon droplets while vegetative cells remained in the bulk aqueous phase. (A) Nonadherent vegetative cell adjacent to small hexadecane droplet covered with adherent endospores (×3200). (B) Endospores adhering to a large hexadecane droplet (×1100). From [13].

Table 1

Bacterial adherence to hydrocarbons: a partial summary of recent investigations

Phenomenon studied	Strains employed	Main findings	References
Effect of growth conditions and phase of growth	*Acinetobacter calcoaceticus* RAG-1; *Serratia marcescens* *Streptococcus pyogenes*	Late-exponential and stationary-phase adhere with higher affinity than exponential-phase cells	8, 19, 27, 28, 29
	A. calcoaceticus RAG-1	Adherence more pronounced in cells grown on complex medium, or on hexadecane, than in ethanol-grown cells	13
	Staphylococcus epidermidis	Cells more hydrophobic following growth on Trypticase soy broth when glucose was present	21
	Various strains	Adherence to hexadecane increased with prolonged starvation in most strains tested	14
	Streptococcus mutans Ingbritt 1600	Decrease in adherence to hexadecane observed for stationary-phase cells	17
	Phormidium strain J-1	Decrease in adherence to hexadecane with increasing culture age	23
Presence of capsule	*A, calcoaceticus* RAG-1 and BD4, *Streptococcus pyogenes*	Capsule interferes with adherence to hexadecane	19, 29
	Coagulase-negative Staphylococci	Most encapsulated strains tested adhered to xylene; large variations observed among non-encapsulated strains	21
Presence of slime	*Pseudomonas aeruginosa*	Clinical isolates of mucoid, slime-producing strains were nonadherent, whereas a non-mucoid non-slime producer adhered with high affinity	30, 31
	Coagulase-negative Staphylococci	Most slime-producing strains adhered poorly to xylene; washing of a slime producer resulted in increased adherence	21
Comparisons of closely related strains	M serotypes of *S. pyogenes* (four tested)	All strains adhered well when harvested from stationary phase	19
	Streptococcus mutans (eight strains tested)	Large variations observed in adherence to hexadecane	17
	Streptococcus salivarius (four strains tested)	All strains tested adhered well to hexadecane	17
	Streptococcus sanguis (five strains tested)	All strains tested adhered well to hexadecane	17
	Streptococcus miteor (three strains tested)	All strains tested adhered well to hexadecane	17
	Staphylococcus epidermidis (fourteen strains tested)	Large variations observed in adherence to xylene	21
	Acinetobacter calcoaceticus (eight strains tested)	Large variations observed in adherence to hexadecane	13
Inhibitory agents	*A. calcoaceticus* RAG-1 and BD413; *S. pyogenes* M-5	Emulsan, an amphipathic polysaccharide produced by RAG-1 cells, inhibits adherence to octane	25
	S. pyogenes	Fatty-acid free bovine serum albumin inhibits adherence to hexadecane	19
	S. mutans	s-IgA inhibits adherence to hydrocarbon	J. Olsson, personal communication

Table 1 (continued)

Phenomenon studied	Strains employed	Main findings	References
Surface components which contribute to adherent properties	*A. calcoaceticus* RAG-1	Thin fimbriae (3 nm diam.) are one of several surface components which promote RAG-1 adherence to hydrocarbons	29
	S. sanguis	Surface fibrils, present on adherent, wild-type cells, are absent on mutant deficient in adherence to hexadecane	16
	S. pyogenes	M protein: lipoteichoic acid complex implicated in hydrophobic surface properties	19
Adherence of rough vs. smooth strains	*Escherichia coli, Proteus mirabilis, Salmonella typhimurium*	Rough mutants more adherent than smooth counterparts	8, 24, 32
Adhesion of benthic Cyanobacteria	various	Benthic strains adhered to hexadecane whereas planktonic strains were nonadherent	23
Growth on hydrocarbon	*A. calcoaceticus* RAG-1	Mutant MR-481, deficient in its ability to adhere to hydrocarbons, was similarly unable to grow on hexadecane under conditions which did not allow for emulsification of hydrocarbon; prolonged incubation of the non-adherent mutant in hexadecane medium enriched for revertants with partial adherence properties	26, 29
Adherence to polystyrene	Various species	Overall correlations observed between adherence to hydrocarbons and adherence to polystyrene	16, 33
Adherence to FEP-teflon	*Staphylococcus epidermidis,*	Correlation between adherence to FEP-teflon and to xylene.	21, 34
Effect of subculturing	*S. mutans*	Serotype C strains progressively lost their ability to adhere to hexadecane with sequential subculturing, whereas serotype d/g strains did not	18
Adherence in oral cavity	155 fresh adherent oral isolates	72% of isolates tested adhered to hexadecane	35
	S. sanguis FC-1	Mutant deficient in adherence to hexadecane was similarly deficient in adherence to saliva-coated hydroxylapatite (s-HA), and in saliva-mediated agglutination	16
	S. sanguis	Correlations between BATH and adherence to s-HA	20
	Various oral laboratory strains	Overall correlation between BATH and adherence to s-HA	15
	A. calcoaceticus RAG-1	Correlations between adherence to hydrocarbons and adherence to buccal epithelial cells and teeth	25, 36, 37
	Dispersed supragingival plaque	The majority of plaque microorganisms obtained and tested directly from the tooth surface adhered to hydrocarbons	37
	S. mutans	Hydrophobic strains implanted better in the oral cavity of volunteers than nonhydrophobic variants	(Svanberg, M., Westergren, G. and Ollson, J., submitted for publication)

role of dextransucrase as a hydrophobic surface factor in *Streptococcus mutans* (R.J. Doyle and J.D. Oakley, personal communication).

5. PROBLEMS AND PROSPECTS

Many unanswered questions remain regarding bacterial hydrophobicity in general and the mechanism and nature of BATH in particular. Several are listed below.

(i) How can the affinities of the various bacteria which do adhere to hydrocarbons be compared? In most studies to date, BATH has been observed to be an all-or-non phenomenon, i.e., cells which do adhere vs. those which do not. However, in some cases [13,25,28,29,32] varying degrees of affinity have been observed. There are at least three ways in which the present assay may be refined to compare the affinities of various hydrophobic strains: (a) If adhering cells do deform the interface, as was suggested by Mudd and Mudd [2], then quantitative determination of the degree of immersion of the cell into the hydrocarbon phase could provide quantitative data on the energy of the interaction [38]; (b) the force required to desorb cells from the interface could be determined in a manner similar to that described by Miura et al. for yeast cells [6]; (c) affinities may be compared by measuring the concentration of surface active agents necessary to inhibit or reverse BATH [25,39].

(ii) Why are different results obtained with various test hydrocarbons? Cells often are observed to adhere better to octane and xylene than to hexadecane [32,37]. Although this question may be related to the viscosity of the test hydrocarbon and size of the droplets formed during the mixing, this point has not been properly studied.

(iii) What is the relationship between BATH and other methods for measuring bacterial hydrophobicity? Although BATH usually correlates with other phenomena of bacterial hydrophobicity (Table 1), some exceptions do exist. For example, *A. calcoaceticus* BD4 cells grown in complex medium adhere to polystyrene but not to hexadecane (N. Kaplan and M. Rosenberg, unpublished observation). Similarly, the reported ability of various strains of *Escherichia coli* to exhibit hydrophobic surface properties as determined by other techniques has not been substantiated by BATH (E.Z. Ron and T. Zusman, unpublished data; I. Winkler, personal communication; [13]). It has recently been suggested that adherence to hydrocarbons requires more pronounced cell surface hydrophobicity than that which can be measured by other techniques [14]. Thus, additional comparisons of the various assays available for determining bacterial hydrophobicity would be valuable.

(iv) How are the surface components which mediate BATH arranged on the cell surface? Very little is known about the localization and distribution of surface components which mediate BATH. In some cases, adherence at the oil : water interface clearly involves a polar orientation [3,4], but in most cases it is not clear whether orientation of the cell at the interface occurs. Similarly, nothing is known about the interfacial orientation of surface projections, such as fimbriae or fibrils, which may play a role in the adherence process [16,28]. Do they spread out along the interface, or poke into the oil phase?

(v) Is adherence to hydrocarbons a property which can be genetically transferred from strain to strain and species to species? Hopefully, this question will be addressed in future research.

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REFERENCES

[1] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 633–645.
[2] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 647–660.

[3] Marshall, K.C. and Cruickshank, R.H. (1973) Arch. Microbiol. 91, 29–40.

[4] Marshall, K.C., Cruickshank, R.H. and Bushby, H.V.A. (1975) J. Gen. Microbiol. 91, 198–200.

[5] Kennedy, R.S., Finnerty, W.R., Sudarsanan, K. and Young, R.A. (1975) Arch. Microbiol. 102, 75–83.

[6] Miura, Y., Okazaki, M., Hamada, S.-I., Murakawa, S.-I. and Yugen, R. (1977) Biotechnol. Bioeng. 19, 701–714.

[7] Kirschner Zilber, I., Rosenberg, E. and Gutnick, D. (1980) Appl. Environ. Microbiol. 40, 1086–1093.

[8] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[9] Blanchard, D.C. and Syzdek, L.D. (1978) Limnol. Oceanogr. 23, 389–400.

[10] Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. (1981) Arch. Microbiol. 128, 267–270.

[11] Kjelleberg, S., Lagercrantz, C. and Larsson, Th. (1980) FEMS Microbiol. Lett. 7, 41–44.

[12] Rosenberg, M., Rosenberg, E. and Gutnick, D. (1980) in Microbial Adhesion to Surfaces (R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent, Eds.), pp. 541–541, Ellis Horwood, Chichester, U.K.

[13] Rosenberg, M. (1982) Ph.D. Thesis, Tel-Aviv University, Ramat-Aviv, Israel.

[14] Kjelleberg, S. and Hermansson, M. (submitted for publication).

[15] Gibbons, R.J. and Etherden, I. (1984) Infect. Immun. (in press).

[16] Gibbons, R.J., Etherden, I. and Skobe, Z. (1983) Infect. Immun. 41, 414–417.

[17] Olsson, J. and Westergren, G. (1982) FEMS Microbiol. Lett. 15, 319–323.

[18] Westergren, G. and Olsson, J. (1983) Infect. Immun. 40, 432–435.

[19] Ofek, I., Whitnack, E. and Beachey, E.H. (1983) J. Bacteriol. 154, 139–145.

[20] Nesbitt, W.E., Doyle, R.J. and Taylor, G. (1982) Infect. Immun. 38, 637–644.

[21] Hogt, A.H., Dankert, J. and Feijen, J. (1983) FEMS Microbiol. Lett. 18, 211–215.

[22] Hogt, A.H., Dankert, J., Feijen, J. and De Vries, J.A. (1982) Antonie van Leeuwenhoek 48, 496–498.

[23] Fattom, A. and Shilo, M. (1984) Appl. Environ. Microbiol. (in press).

[24] Bohach, G.A. and Snyder, I.S. (1983) Infect. Immun. 42, 318–325.

[25] Rosenberg, E., Gottlieb, A. and Rosenberg, M. (1983) Infect. Immun. 39, 1024–1028.

[26] Rosenberg, M. and Rosenberg, E. (1981) J. Bacteriol. 148, 51–57.

[27] Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) Infect. Immun. 33, 29–33.

[28] Rosenberg, M., Bayer, E.A., Delarea, J. and Rosenberg, E. (1982) Appl. Environ. Microbiol. 44, 929–937.

[29] Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M. and Gutnick, D. (1983) FEMS Microbiol. Lett. 17, 157–160.

[30] Meshulam, T. (1983) D.Sc. Thesis, Technion—Israel Institute of Technology, Haifa, Israel.

[31] Merzbach, D., Obedenu, N., Meshulam, T. and Sobel, J.D. (1982) Isr. J. Med. Sci. 18, 5.

[32] Rosenberg, M., Rottem, S. and Rosenberg, E. (1982) FEMS Microbiol. Lett. 13, 167–169.

33 Rosenberg, M. (1981) Appl. Environ. Microbiol. 42, 375–377.

[34] Hogt, A.H., Dankert, J., De Vries, J.A. and Feijen, J. (1983) J. Gen. Microbiol. 129, 2959–2968.

[35] Weiss, E., Rosenberg, M., Judes, H. and Rosenberg, E. (1982) Curr. Microbiol. 7, 125–128.

[36] Weiss, E., Judes, H. and Rosenberg, M. (1984) Dent. Med. (in press).

[37] Rosenberg, M., Judes, H. and Weiss, E. (1983) Infect. Immun. 42, 831–834.

[38] Gingell, D. and Todd, I. (1975) J. Cell Sci. 18, 227–239.

[39] Neumann, A.W., Absolom, D.R., Van Oss, C.J. and Zingg, W. (1979) Cell Biophys. 1, 79–92.

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Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene

(Hydrophobicity; hexadecane; polystyrene; adhesion; cell surface)

Mel Rosenberg

School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel

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1. SUMMARY

Bacterial adherence to hydrocarbons (BATH) is a convenient method for the study of relatively pronounced cell surface hydrophobicity exhibited by various microbial cells, but has proven relatively ineffective in the study of less pronounced examples of bacterial hydrophobicity. It is well known that hydrophobic interactions can be enhanced by addition of salting-out agents, such as ammonium sulphate. In the present manuscript, the effect of ammonium sulphate on adherence to hexadecane of *Escherichia coli* strains K-12 and J-5 was studied. In the absence of ammonium sulphate, neither strain adhered to hexadecane. However, adherence of rough strain J-5 was enhanced dramatically in the presence of rising ammonium sulphate concentrations. Ammonium sulphate similarly enhanced adherence to other test hydrocarbons, and to the solid hydrophobic surface, polystyrene. The results show that the presence of ammonium sulphate can promote bacterial adherence to hydrocarbon and thus extend the use of this technique to include a wider array of microorganisms.

2. INTRODUCTION

Bacterial hydrophobicity is currently recognized as an important factor in mediating a wide variety of bacterial adherence phenomena. In recent years, BATH has proven to be a convenient technique for assaying cell surface hydrophobicity of many hydrophobic bacterial strains [1,2]. One of the shortcomings of this method is its relative insensitivity in measuring less pronounced examples of cell surface hydrophobicity [1]. For example, increased cell surface hydrophobicity has been reported for *E. coli* strains bearing various fimbriae [3–8] as measured by hydrophobic interaction chromatography (HIC); however, such strains generally fail to adhere appreciably to hydrocarbons, using the BATH assay as originally described [9]. One striking difference between the two techniques is that salting-out agents, e.g. ammonium sulphate are usually employed using HIC, whereas they are not added in the basic BATH assay.

In the present study, the possibility that ammonium sulphate can similarly promote adherence to hydrocarbons was tested, using *E. coli* K-12 and the rough mutant strain, J-5 [9]. The dramatic

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increase in adherence of the latter strain to hexadecane upon raising the ammonium sulphate concentration suggests that such a modified procedure may be useful in measuring differences in cell surface hydrophobicity which are not readily distinguishable using the original technique. The increased adherence of strain J-5 to hydrocarbons in the presence of ammonium sulphate was paralleled by a similar increase in adherence to polystyrene.

3. MATERIALS AND METHODS

E. coli K-12 (CSH 57) and J-5, a rough mutant strain derived from *E. coli* K-12, were originally obtained from E.Z. Ron [9]. For adherence experiments, cells were inoculated 1 : 200 and grown for 18 h on brain heart infusion broth (Difco) at 30 °C with shaking. Cells were washed twice and suspended in phosphate buffer (22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, and distilled water to 1 liter, pH 7.1).

3.1. Adherence to hydrocarbon

Cells were suspended in phosphate buffer to a corrected absorbance at 400 nm of 3.0, as measured in a Uvikon 710 spectrophotometer (Kontron, Zurich, Switzerland). To 0.4 ml of cell suspension in acid-washed, standard 10-mm dia test tubes, 0.8 ml of phosphate buffer containing various three-fold concentrations of ammonium sulphate, or alternatively, phosphate buffer alone, were added. To each test tube, 0.2 ml of test hydrocarbon (*n*-hexadecane, *n*-octane or *p*-xylene) were added, and following preincubation at 30 °C [9], test tubes were uniformly vortexed on a Thermolyne Maxi Mix (Sybron, Dubuque, Iowa) for 120 s. Immediately following phase separation, the bottom aqueous phase was removed with a Pasteur pipette and carefully transferred to a disposable 1 ml cuvette. Light absorbance was determined at 400 nm. Adherence is expressed as the percent decrease in turbidity following the mixing procedure, as compared to that of the corresponding cell suspension prior to the assay. It should be emphasized that changes of the order of 10–20% were observed in the initial turbidity of the cell suspensions when ammonium sulphate was present, as previously reported by Smyth et al. [3], necessitating measurement of the initial turbidity of each sample.

3.2. Adherence to polystyrene

Cells were suspended either in phosphate buffer, or in phosphate buffer containing 2 M ammonium sulphate to an absorbance at 400 nm of approx. 3.5. 50-μl aliquots of each cell suspension were added to 40 wells in a flat-bottom microtiter plate (non-treated polystyrene, NUNC, Roskilde, Denmark). Following 20 min incubation at room temperature, wells were washed with the corresponding suspending solution (i.e., phosphate buffer, or phosphate buffer containing 2 M ammonium sulphate), stained with Gentian violet [10] and allowed to dry. Results are expressed as absorbance at 570 nm, measured in a Dynatech MR600 microplate reader.

4. RESULTS

Fig. 1 demonstrates the effect of rising ammonium sulphate concentrations on the adherence of *E. coli* J-5 and K-12 to hexadecane. Both strains were completely nonadherent when ammonium sulphate was absent from the assay mixture. Similarly, no adherence of strain K-12 was observed when ammonium sulphate was present in the assay. In contrast, adherence of rough mutant J-5 [9] to hexadecane rose dramatically with increasing concentrations of ammonium sulphate, reaching 76% at 2 M. Adherence of J-5 cells at the aqueous: oil interface was confirmed by microscopic observation of the hydrocarbon droplets in the upper phase following the assay procedure.

Adherence of both strains to *n*-octane and *p*-xylene, in the absence and presence of 2 M ammonium sulphate, is shown in Table 1. In each case, adherence rose in the presence of the salting out agent. Moreover, the extent of adherence of J-5 was in each case greater than that of K-12, although the latter strain did exhibit significant adherence to xylene, in the presence of ammonium sulphate.

The ability of the two strains to adhere to the hydrophobic solid surface, polystyrene [10], in the

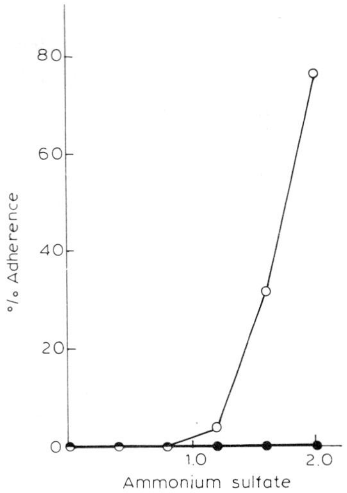

Fig. 1. Adherence to hexadecane as a function of ammonium sulphate concentration. Cells of *E. coli* K-12 (●) and J-5 (○) were grown, harvested, washed and suspended in phosphate buffer as described in the text. Adherence to 0.2 ml hexadecane in the presence of varying concentrations of ammonium sulphate was performed as described in MATERIALS AND METHODS. Results are shown as the % decrease in turbidity, following the assay procedure, as compared to the initial turbidity of the particular sample, as a function of ammonium sulphate concentration.

presence and absence of ammonium sulphate, is compared in Table 2. In the absence of ammonium sulphate, both strains adhered poorly to the plastic surface. However, in the presence of ammonium sulphate, adherence of J-5 increased significantly.

Table 1

Adherence to *n*-octane and *p*-xylene [a]

Strain	Ammonium sulphate (2 M final concentration)	% adherence to	
		n-Octane	*p*-Xylene
K-12	−	0.8	0
	+	11	67
J-5	−	0.7	19
	+	37	82

[a] Cells were grown, harvested, washed and suspended as described in the text. Adherence to 0.2 ml of *n*-octane or *p*-xylene was performed as described in MATERIALS AND METHODS, in the absence or presence of 2 M ammonium sulphate. Results are expressed as the % decrease in turbidity as compared with that prior to the mixing procedure.

Table 2

Adherence to polystyrene [a]

Strain	Ammonium sulphate	Absorbance ($x \pm$ S.D.)
K-12	−	0.018 ± 0.004
	+	0.023 ± 0.006
J-5	−	0.026 ± 0.004
	+	0.048 ± 0.013

[a] Cells were grown, washed and suspended as described in the text. Adherence to polystyrene microtiter wells was performed as described in MATERIALS AND METHODS, in the absence or presence of 2 M ammonium sulphate. Results are expressed as mean absorbance at 570 nm.

5. DISCUSSION

The ability of certain bacteria to partition at the aqueous : oil interface, and the relationship between this phenomenon and cell-surface hydrophobicity were documented 60 years ago in the work of Mudd and Mudd [11,12], and more recently by Marshall and coworkers [13,14]. Several years ago BATH was suggested as a simple quantitative method for measuring bacterial hydrophobicity [9]. This technique has enabled demonstration of cell surface hydrophobicity in a variety of bacterial strains [9], and cell mixtures [1,15], and has been employed to show correlations between bacterial hydrophobicity and other biological phenomena [1], as well as for mutant isolation [16].

One area in which BATH has failed to make a significant contribution is that of differences in cell surface hydrophobicity among strains which adhere very poorly or not at all to hydrocarbon under the original conditions of the assay [1,9]. The purpose of the present paper was to test the ability of ammonium sulphate to increase the sensitivity of BATH with respect to such strains, similar to its reported effect in stimulating adherence of enerobacterial strains to hydrophobic Sepharose beads [3–8,17–19]. In the absence of ammonium sulphate, adherence of *E. coli* K-12 and rough mutant J-5 to hexadecane was zero, under the growth conditions employed. Performance of the assay in rising concentrations of ammonium sulphate led to a dramatic increase in adherence of J-5, whereas K-12 remained completely nonadherent. These results are consistent with the very slight increase in adherence to hydrocarbons previously reported for J-5 vs. K-12 [9], and for increased hydrophobicity of other rough mutants reported using various techniques [20,21]. Less dramatic differences in adherence of the two strains to octane and xylene were found, suggesting that hexadecane may be preferable when this modification is employed. Nevertheless, ammonium sulphate promoted adherence levels in each case.

Increased adherence of J-5 to polysterene was similarly recorded in the presence of ammonium sulphate, whereas adherence of K-12 was relatively unaffected. Good correlations have been previously reported for bacterial adherence to hexadecane and polystyrene [10], with the occasional exception [1].

As has been previously pointed out [1,22], the mixing conditions employed can affect the results obtained using BATH. Similarly, in the present study, greater adherence to hexadecane could be obtained at lower ammonium sulphate concentrations by stronger agitation (not shown). Thus, when using BATH, or the present modification, all assay conditions should be kept identical.

There is, at present, some confusion over the role played by hydrophobic interactions in mediating the adherence properties of enterobacterial cells. For example, do hydrophobic interactions contribute to more specific lectin-like bonds between bacterium and substratum, as has been proposed for mannose-sensitive *E. coli* strains [3,5,19]? What is the degree, extent and role of cell surface hydrophobicity in mediating adherence of 'mannose-resistant' strains? Can enterobacterial strains be ranked in increasing order of cell-surface hydrophobicity, according to various techniques? Finally, how does the cell-surface hydrophobicity reported for such enterobacterial cells compare to that observed for other types of microorganisms, such as oral bacteria [15,22]?

Since all the methods for measuring bacterial hydrophobicity differ somewhat in the precise properties which they measure [9], the provision of additional techniques and extension of existing methods may prove advantageous. The modification of BATH and adherence to polystyrene proposed here, based on addition of ammonium sulphate, may extend the use of these two simple techniques to include a wider array of microbial cells.

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REFERENCES

[1] Rosenberg, M. (1984) FEMS Microbiol. Lett. 22, 289–295.

[2] Rosenberg, M., Rosenberg, E., Judes, H. and Weiss, E. (1983) FEMS Microbiol. Lett. 20, 1–5.

[3] Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, T. (1978) Infect. Immun. 22, 462–472.

[4] Lindahl, M., Farias, A., Wadström, T. and Hjertén, S. (1981) Biochim. Biophys. Acta 677, 471–476.

[5] Jann, K., Schmidt, G., Blumenstock, E. and Vosbeck, K. (1981) Infect. Immun. 32, 484–489.

[6] Öhman, L., Normann, B. and Stendahl, O. (1981) Infect. Immun. 32, 951–955.

[7] Öhman, L., Magnusson, K.-E. and Stendahl, O. (1982) FEMS Microbiol. Lett. 14, 149–153.

[8] Faris, A., Wadström, T. and Freer, J.H. (1981) Curr. Microbiol. 5, 67–72.

[9] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[10] Rosenberg, M. (1981) Appl. Environ. Microbiol. 42, 375–377.

[11] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 633–645.

[12] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 647–660.

[13] Marshall, K.C. and Cruickshank, R.H. (1973) Arch. Microbiol. 91, 29–40.

[14] Marshall, K.C., Cruickshank, R.H. and Bushby, H.V.A. (1975) J. Gen. Microbiol. 91, 198–200.

[15] Rosenberg, M., Judes, H. and Weiss, E. (1983) Infect. Immun. 42, 831–834.

[16] Rosenberg, M. and Rosenberg, E. (1981) J. Bacteriol. 148, 51–57.

[17] Martinez, R.J. (1983) Infect. Immun. 41, 921–930.

[18] Jonsson, P. and Wadström, T. (1983) Curr. Microbiol. 8, 347–354.

[19] Öhman, L. (1983) Ph.D. Thesis, Linkoping University, Linkoping, Sweden.

[20] Edebo, L., Kihlstrom, E., Magnusson, K.-E. and Stendahl, O. (1980) in Cell Adhesion and Motility (Curtis, A.S.G. and Pitts, J.D., Eds.), pp. 65–103. Cambridge University Press, New York.

[21] Rosenberg, M., Rottem, S. and Rosenberg, E. (1982) FEMS Microbiol. Lett. 13, 167–169.

[22] Gibbons, R.J. and Etherden, I. (1983) Infect. Immun. 41, 1190–1196.

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Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity

(Hydrophobicity; adherence; hydrocarbons; bacteria; cell-surface; method)

Mel Rosenberg

School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel

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1. SUMMARY

Bacterial adherence to hydrocarbons (BATH) is a simple and rapid technique for determining cell-surface hydrophobicity. During recent years, this method has found application in the study of the surface characteristics of a wide variety of bacteria and bacterial mixtures. Correlations have been found between the adherence of bacteria to hydrocarbons and their attachment to other surfaces, including non-wettable plastics, epithelial cells, and teeth. A slight modification of the assay enables the isolation of nonhydrophobic mutants. The present publication briefly describes the technique and its modifications, summarizes results obtained using this method, and suggests several directions for further investigation.

2. INTRODUCTION

Bacterial adherence to oils was observed and documented in the pioneering studies of Mudd and Mudd [1,2] some 60 years ago, and more recently, in reports by K.C. Marshall and co-workers, on the polar adhesion of *Hyphomicrobium, Flexibacter* and *Rhizobium* strains at the oil : water interface [3,4]. Despite these and other published data, researchers in the field of petroleum microbiology have often considered adherence to oil to be a specific property of oil-degrading microorganisms [5–7].

Our interest in the possible role of adherence in growth of the marine bacterium, *Acinetobacter calcoaceticus* RAG-1, on hexadecane led us to develop a simple quantitative assay to test this question [8]. Initial results showed that (i) although RAG-1 adheres avidly to hydrocarbons which it metabolizes (e.g. hexadecane), it adheres just as well to other hydrocarbons which it cannot degrade [8], and (ii) certain other bacteria, such as *Serratia marcescens,* which are known to possess hydrophobic surface properties [9–14], adhere avidly to hydrocarbons, despite their inability to degrade them [8]. These results led us to suggest that bacterial adherence to hydrocarbons (BATH) could serve as a general, quantitative assay for measuring cell-surface hydrophobicity [8,12,13]. Since then, BATH has been employed in a variety of investigations concerned with the microbial cell surface.

The purpose of the present manuscript is (i) to describe aspects pertaining to the technique itself; (ii) to present some of the results obtained employing BATH, and (iii) to suggest several avenues for further investigation.

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BATH—METHODOLOGICAL ASPECTS

Details of the basic technique for measuring bacterial adherence to hydrocarbons have been described [8,12,13], and can be summarized as follows: a turbid, aqueous suspension of washed microbial cells is mixed by vortexing in the presence of a test liquid hydrocarbon under controlled conditions. Following mixing, the two phases are allowed to separate. In the case of adherence, cells from the bulk aqueous phase become bound to hydrocarbon droplets and rise with them following the mixing to form an upper 'cream' consisting of cell-coated oil droplets. When nonadherent bacteria are tested, the phases separate following the mixing procedure, with the cells remaining in the bulk aqueous suspension. The percentage of adherent cells can easily be ascertained by the decrease in absorbance of the lower aqueous phase following the assay, as compared to the absorbance of the original bacterial suspension. Alternatively, the cells may be radiolabelled, and the radioactivity remaining in the aqueous phase measured [11]. It is also possible to perform viable counts prior to and following the mixing procedure, provided that proper controls are undertaken (E. Rosenberg, personal communication).

Various details which were originally employed in the paper describing the assay have since been modified and amended in various investigations. Parameters which have been altered include the suspending buffer [14,17–23], the volumes and ratios of the phases employed [15–21,23,24], the test tubes used [15,19,20,23,24], duration of mixing [15,16,18–21,23,24], and the test hydrocarbons employed [20]. Results obtained in this and other laboratories have shown that several aspects of the assay appear to be of major importance:

(i) The aqueous bacterial suspension must be free of surfactants, which inhibit the assay [8,21–23,25]. Similarly, the test tubes must be uniformly clean, preferably acid-washed [8].

(ii) Both the lower aqueous phase and the upper 'cream' should be examined microscopically to ensure that the cells are indeed at the hydrocarbon : water interface, and that the decrease in turbidity is not due to some other factor, such as clumping or lysis.

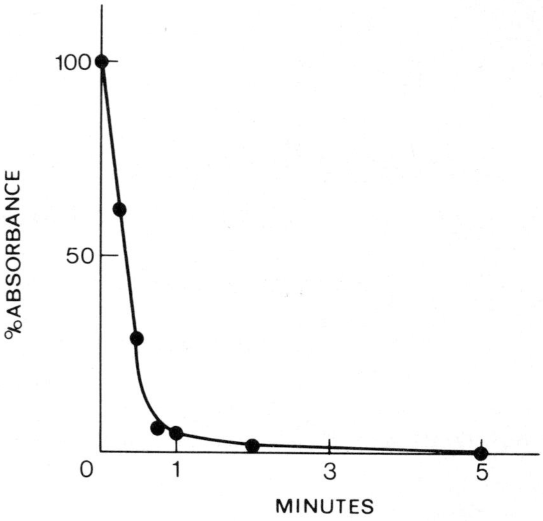

Fig. 1. Adherence to hexadecane as a function of duration of the mixing procedure. *Acetinetobacter calcoaceticus* RAG-1 cells were grown to early stationary phase on nutrient broth, washed and suspended in PUM buffer [8] to an initial absorbance at 400 nm of 1.48. To 1.2 ml aqueous cell suspension, 0.2 ml hexadecane were added and the mixtures vortexed for different lengths of time. All other assay conditions were as described in [8].

(iii) The assay can be 'overloaded' if the initial bacterial suspension is too concentrated. Thus, various bacterial concentrations should be assayed at the outset of any investigation.

(iv) Insufficient mixing may not allow for maximal bacterial adherence. This can be easily ascertained by increasing the duration of the vortexing procedure (Fig. 1 [13]). A flat-top mixer (such as the Multitube vortexer, SMI, or the Thermolyne maxi mix, Sybron) is preferable to single-tube vortexers, in that a number of tubes can be mixed simultaneously.

(v) The ionic strength and composition of the buffer employed may alter results. Several investigators have reported decreased adherence as a function of lowering the ionic strength of the suspending buffer [19,20,23].

(vi) Since the adherence of a given strain may largely depend on the growth conditions employed [8,11,13,17,21,23,27], adherence should be checked at various phases of growth, at different growth temperatures, and following growth on different media before proclaiming a given strain to be 'nonadherent'.

3.1. Mutant isolation and cell separations

Under optimal conditions, certain bacterial strains have been found to adhere to hydrocarbons to the extent of approx. 95% or more. In such cases, a slight modification of the adherence assay yields an enrichment technique for the isolation of nonhydrophobic mutants [26]. In this procedure, washed wild-type cells are vortexed in the presence of hexadecane or octane under sterile conditions. The lower aqueous phase is then removed and again extracted with hydrocarbon until the cells remaining in the aqueous phase are too few to be seen under microscopic examination. The lower aqueous phase is then inoculated into growth medium. Following growth, the cells are harvested, washed, and again mixed with the hydrocarbon as before. Following several extractions, the clear lower aqueous phase is once again inoculated into growth medium. Following several growth and extraction cycles, it may be observed that the test hydrocarbon is no longer able to extract the vast majority of cells. At this stage the lower aqueous phase is plated out and individual clones are assayed for BATH. Thus far, this technique has enabled isolation of nonhydrophobic mutants of *Acinetobacter calcoaceticus* [26], *Streptococcus sanguis* [16], and *Serratia marcescens* (M. Rosenberg, unpublished data). It would be of interest to determine whether other adherent strains yield nonhydrophobic mutants following this procedure.

BATH may also be employed to separate hydrophobic and hydrophilic cell mixtures (e.g., hydrophobic spores from vegetative cells), as shown in Fig. 2.

4. RESULTS OBTAINED USING BATH

A summary of some recent investigations which employed BATH, and the main results obtained, is presented in Table 1. Several lines of research which use this technique are currently in progress. These include studies on gliding motility in *Cytophaga johnsonae* (R. Wolkin, personal communication), swarming in *Proteus mirabilis* (Gail Johnston, personal communication), the relationship between phase variation in *Bordetella pertussis* and cell-surface hydrophobicity (F. Fish and S. Goldman, personal communication), the effect of specific growth rate on adherence and hydrophobicity of oral *Actinomyces* (R.P. Ellen and I. Hamilton, unpublished results), and the possible

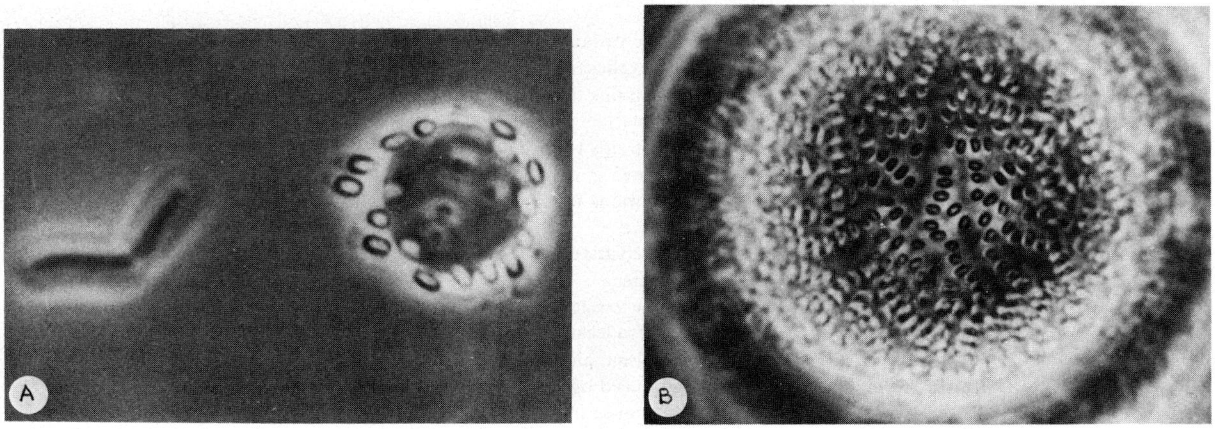

Fig. 2. Adherence of *Bacillus cereus* endospores to hexadecane. When a mixture of vegetative and endospore cells of *B. cereus*, scraped from a nutrient agar plate following 72 h incubation and suspended in PUM buffer [8], was mixed in the presence of hexadecane, spore cells adhered to the hydrocarbon droplets while vegetative cells remained in the bulk aqueous phase. (A) Nonadherent vegetative cell adjacent to small hexadecane droplet covered with adherent endospores (×3200). (B) Endospores adhering to a large hexadecane droplet (×1100). From [13].

Table 1

Bacterial adherence to hydrocarbons: a partial summary of recent investigations

Phenomenon studied	Strains employed	Main findings	References
Effect of growth conditions and phase of growth	*Acinetobacter calcoaceticus* RAG-1; *Serratia marcescens* *Streptococcus pyogenes*	Late-exponential and stationary-phase adhere with higher affinity than exponential-phase cells	8, 19, 27, 28, 29
	A. calcoaceticus RAG-1	Adherence more pronounced in cells grown on complex medium, or on hexadecane, than in ethanol-grown cells	13
	Staphylococcus epidermidis	Cells more hydrophobic following growth on Trypticase soy broth when glucose was present	21
	Various strains	Adherence to hexadecane increased with prolonged starvation in most strains tested	14
	Streptococcus mutans Ingbritt 1600	Decrease in adherence to hexadecane observed for stationary-phase cells	17
	Phormidium strain J-1	Decrease in adherence to hexadecane with increasing culture age	23
Presence of capsule	*A, calcoaceticus* RAG-1 and BD4, *Streptococcus pyogenes*	Capsule interferes with adherence to hexadecane	19, 29
	Coagulase-negative Staphylococci	Most encapsulated strains tested adhered to xylene; large variations observed among non-encapsulated strains	21
Presence of slime	*Pseudomonas aeruginosa*	Clinical isolates of mucoid, slime-producing strains were nonadherent, whereas a non-mucoid non-slime producer adhered with high affinity	30, 31
	Coagulase-negative Staphylococci	Most slime-producing strains adhered poorly to xylene; washing of a slime producer resulted in increased adherence	21
Comparisons of closely related strains	M serotypes of *S. pyogenes* (four tested)	All strains adhered well when harvested from stationary phase	19
	Streptococcus mutans (eight strains tested)	Large variations observed in adherence to hexadecane	17
	Streptococcus salivarius (four strains tested)	All strains tested adhered well to hexadecane	17
	Streptococcus sanguis (five strains tested)	All strains tested adhered well to hexadecane	17
	Streptococcus miteor (three strains tested)	All strains tested adhered well to hexadecane	17
	Staphylococcus epidermidis (fourteen strains tested)	Large variations observed in adherence to xylene	21
	Acinetobacter calcoaceticus (eight strains tested)	Large variations observed in adherence to hexadecane	13
Inhibitory agents	*A. calcoaceticus* RAG-1 and BD413; *S. pyogenes* M-5	Emulsan, an amphipathic polysaccharide produced by RAG-1 cells, inhibits adherence to octane	25
	S. pyogenes	Fatty-acid free bovine serum albumin inhibits adherence to hexadecane	19
	S. mutans	s-IgA inhibits adherence to hydrocarbon	J. Olsson, personal communication

Table 1 (continued)

Phenomenon studied	Strains employed	Main findings	References
Surface components which contribute to adherent properties	*A. calcoaceticus* RAG-1	Thin fimbriae (3 nm diam.) are one of several surface components which promote RAG-1 adherence to hydrocarbons	29
	S. sanguis	Surface fibrils, present on adherent, wild-type cells, are absent on mutant deficient in adherence to hexadecane	16
	S. pyogenes	M protein : lipoteichoic acid complex implicated in hydrophobic surface properties	19
Adherence of rough vs. smooth strains	*Escherichia coli, Proteus mirabilis, Salmonella typhimurium*	Rough mutants more adherent than smooth counterparts	8, 24, 32
Adhesion of benthic Cyanobacteria	various	Benthic strains adhered to hexadecane whereas planktonic strains were nonadherent	23
Growth on hydrocarbon	*A. calcoaceticus* RAG-1	Mutant MR-481, deficient in its ability to adhere to hydrocarbons, was similarly unable to grow on hexadecane under conditions which did not allow for emulsification of hydrocarbon; prolonged incubation of the non-adherent mutant in hexadecane medium enriched for revertants with partial adherence properties	26, 29
Adherence to polystyrene	Various species	Overall correlations observed between adherence to hydrocarbons and adherence to polystyrene	16, 33
Adherence to FEP-teflon	*Staphylococcus epidermidis,*	Correlation between adherence to FEP-teflon and to xylene.	21, 34
Effect of subculturing	*S. mutans*	Serotype C strains progressively lost their ability to adhere to hexadecane with sequential subculturing, whereas serotype d/g strains did not	18
Adherence in oral cavity	155 fresh adherent oral isolates	72% of isolates tested adhered to hexadecane	35
	S. sanguis FC-1	Mutant deficient in adherence to hexadecane was similarly deficient in adherence to saliva-coated hydroxylapatite (s-HA), and in saliva-mediated agglutination	16
	S. sanguis	Correlations between BATH and adherence to s-HA	20
	Various oral laboratory strains	Overall correlation between BATH and adherence to s-HA	15
	A. calcoaceticus RAG-1	Correlations between adherence to hydrocarbons and adherence to buccal epithelial cells and teeth	25, 36, 37
	Dispersed supragingival plaque	The majority of plaque microorganisms obtained and tested directly from the tooth surface adhered to hydrocarbons	37
	S. mutans	Hydrophobic strains implanted better in the oral cavity of volunteers than nonhydrophobic variants	(Svanberg, M., Westergren, G. and Ollson, J., submitted for publication)

role of dextransucrase as a hydrophobic surface factor in *Streptococcus mutans* (R.J. Doyle and J.D. Oakley, personal communication).

5. PROBLEMS AND PROSPECTS

Many unanswered questions remain regarding bacterial hydrophobicity in general and the mechanism and nature of BATH in particular. Several are listed below.

(i) How can the affinities of the various bacteria which do adhere to hydrocarbons be compared? In most studies to date, BATH has been observed to be an all-or-non phenomenon, i.e., cells which do adhere vs. those which do not. However, in some cases [13,25,28,29,32] varying degrees of affinity have been observed. There are at least three ways in which the present assay may be refined to compare the affinities of various hydrophobic strains: (a) If adhering cells do deform the interface, as was suggested by Mudd and Mudd [2], then quantitative determination of the degree of immersion of the cell into the hydrocarbon phase could provide quantitative data on the energy of the interaction [38]; (b) the force required to desorb cells from the interface could be determined in a manner similar to that described by Miura et al. for yeast cells [6]; (c) affinities may be compared by measuring the concentration of surface active agents necessary to inhibit or reverse BATH [25,39].

(ii) Why are different results obtained with various test hydrocarbons? Cells often are observed to adhere better to octane and xylene than to hexadecane [32,37]. Although this question may be related to the viscosity of the test hydrocarbon and size of the droplets formed during the mixing, this point has not been properly studied.

(iii) What is the relationship between BATH and other methods for measuring bacterial hydrophobicity? Although BATH usually correlates with other phenomena of bacterial hydrophobicity (Table 1), some exceptions do exist. For example, *A. calcoaceticus* BD4 cells grown in complex medium adhere to polystyrene but not to hexadecane (N. Kaplan and M. Rosenberg, unpublished observation). Similarly, the reported ability of various strains of *Escherichia coli* to exhibit hydrophobic surface properties as determined by other techniques has not been substantiated by BATH (E.Z. Ron and T. Zusman, unpublished data; I. Winkler, personal communication; [13]). It has recently been suggested that adherence to hydrocarbons requires more pronounced cell surface hydrophobicity than that which can be measured by other techniques [14]. Thus, additional comparisons of the various assays available for determining bacterial hydrophobicity would be valuable.

(iv) How are the surface components which mediate BATH arranged on the cell surface? Very little is known about the localization and distribution of surface components which mediate BATH. In some cases, adherence at the oil : water interface clearly involves a polar orientation [3,4], but in most cases it is not clear whether orientation of the cell at the interface occurs. Similarly, nothing is known about the interfacial orientation of surface projections, such as fimbriae or fibrils, which may play a role in the adherence process [16,28]. Do they spread out along the interface, or poke into the oil phase?

(v) Is adherence to hydrocarbons a property which can be genetically transferred from strain to strain and species to species? Hopefully, this question will be addressed in future research.

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REFERENCES

[1] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 633–645.

[2] Mudd, S. and Mudd, E.B.H. (1924) J. Exp. Med. 40, 647–660.

[3] Marshall, K.C. and Cruickshank, R.H. (1973) Arch. Microbiol. 91, 29–40.

[4] Marshall, K.C., Cruickshank, R.H. and Bushby, H.V.A. (1975) J. Gen. Microbiol. 91, 198–200.

[5] Kennedy, R.S., Finnerty, W.R., Sudarsanan, K. and Young, R.A. (1975) Arch. Microbiol. 102, 75–83.

[6] Miura, Y., Okazaki, M., Hamada, S.-I., Murakawa, S.-I. and Yugen, R. (1977) Biotechnol. Bioeng. 19, 701–714.

[7] Kirschner Zilber, I., Rosenberg, E. and Gutnick, D. (1980) Appl. Environ. Microbiol. 40, 1086–1093.

[8] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[9] Blanchard, D.C. and Syzdek, L.D. (1978) Limnol. Oceanogr. 23, 389–400.

[10] Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. (1981) Arch. Microbiol. 128, 267–270.

[11] Kjelleberg, S., Lagercrantz, C. and Larsson, Th. (1980) FEMS Microbiol. Lett. 7, 41–44.

[12] Rosenberg, M., Rosenberg, E. and Gutnick, D. (1980) in Microbial Adhesion to Surfaces (R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent, Eds.), pp. 541–541, Ellis Horwood, Chichester, U.K.

[13] Rosenberg, M. (1982) Ph.D. Thesis, Tel-Aviv University, Ramat-Aviv, Israel.

[14] Kjelleberg, S. and Hermansson, M. (submitted for publication).

[15] Gibbons, R.J. and Etherden, I. (1984) Infect. Immun. (in press).

[16] Gibbons, R.J., Etherden, I. and Skobe, Z. (1983) Infect. Immun. 41, 414–417.

[17] Olsson, J. and Westergren, G. (1982) FEMS Microbiol. Lett. 15, 319–323.

[18] Westergren, G. and Olsson, J. (1983) Infect. Immun. 40, 432–435.

[19] Ofek, I., Whitnack, E. and Beachey, E.H. (1983) J. Bacteriol. 154, 139–145.

[20] Nesbitt, W.E., Doyle, R.J. and Taylor, G. (1982) Infect. Immun. 38, 637–644.

[21] Hogt, A.H., Dankert, J. and Feijen, J. (1983) FEMS Microbiol. Lett. 18, 211–215.

[22] Hogt, A.H., Dankert, J., Feijen, J. and De Vries, J.A. (1982) Antonie van Leeuwenhoek 48, 496–498.

[23] Fattom, A. and Shilo, M. (1984) Appl. Environ. Microbiol. (in press).

[24] Bohach, G.A. and Snyder, I.S. (1983) Infect. Immun. 42, 318–325.

[25] Rosenberg, E., Gottlieb, A. and Rosenberg, M. (1983) Infect. Immun. 39, 1024–1028.

[26] Rosenberg, M. and Rosenberg, E. (1981) J. Bacteriol. 148, 51–57.

[27] Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) Infect. Immun. 33, 29–33.

[28] Rosenberg, M., Bayer, E.A., Delarea, J. and Rosenberg, E. (1982) Appl. Environ. Microbiol. 44, 929–937.

[29] Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M. and Gutnick, D. (1983) FEMS Microbiol. Lett. 17, 157–160.

[30] Meshulam, T. (1983) D.Sc. Thesis, Technion—Israel Institute of Technology, Haifa, Israel.

[31] Merzbach, D., Obedenu, N., Meshulam, T. and Sobel, J.D. (1982) Isr. J. Med. Sci. 18, 5.

[32] Rosenberg, M., Rottem, S. and Rosenberg, E. (1982) FEMS Microbiol. Lett. 13, 167–169.

33 Rosenberg, M. (1981) Appl. Environ. Microbiol. 42, 375–377.

[34] Hogt, A.H., Dankert, J., De Vries, J.A. and Feijen, J. (1983) J. Gen. Microbiol. 129, 2959–2968.

[35] Weiss, E., Rosenberg, M., Judes, H. and Rosenberg, E. (1982) Curr. Microbiol. 7, 125–128.

[36] Weiss, E., Judes, H. and Rosenberg, M. (1984) Dent. Med. (in press).

[37] Rosenberg, M., Judes, H. and Weiss, E. (1983) Infect. Immun. 42, 831–834.

[38] Gingell, D. and Todd, I. (1975) J. Cell Sci. 18, 227–239.

[39] Neumann, A.W., Absolom, D.R., Van Oss, C.J. and Zingg, W. (1979) Cell Biophys. 1, 79–92.

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A kinetic approach to bacterial adherence to hydrocarbon

Dov Lichtenberg[a], Mel Rosenberg[b,c,*], Nellie Sharfman[b] and Itzhak Ofek[b]

[a] *Department of Physiology and Pharmacology,* [b] *Department of Human Microbiology, and* [c] *School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat Aviv 69978 (Israel)*

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Summary

Although bacterial adherence to hydrocarbons has been widely employed to study bacterial hydrophobicity, quantitative criteria for the assay are not well established. In the present report, adherence of *Streptococcus pyogenes* to hexadecane was studied as a function of time, and water: hexadecane ratio. Adherence of cells as a function of mixing time was exponential, yielding a slope for each hexadecane volume employed. The values for slopes of the curves obtained increased linearly with increasing hydrocarbon: water ratios. The slope of the latter curve, referred to as the 'removal coefficient' was 376 min^{-1} for *S. pyogenes*. The data suggest that the proposed kinetic approach may provide a quantitative measurement of bacterial adherence to hydrocarbons.

Key words: *Adherence – Cell surface – Hexadecane – Hydrocarbons – Hydrophobicity* – Streptococcus pyogenes

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Introduction

The ability of certain bacteria to partition at the oil: water interface has been known for over 60 years [1]. Several years ago bacterial adherence to hydrocarbons was proposed as a convenient technique for studying hydrophobic surface properties of microbial cells [2]. In the assay, washed bacterial suspensions are vortexed in the presence of test hydrocarbons (e.g., hexadecane, octane, xylene). Following separation of the phases, which enables cell-coated oil droplets to rise, the extent of adherence is determined from the decrease in cell density in the lower aqueous phase, observed through turbidimetric measurements. During the past few years, this method has been applied to the investigations of bacterial hydrophobicity in a wide range of species, and as a function of various bacterial growth conditions and treatments [3, 4]. Moreover, this assay

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[*] To whom correspondence should be addressed.

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has helped elucidate the possible role of hydrophobic interactions in mediating bacterial adherence to surfaces of ecological and medical interest [3] and for isolation of mutants deficient in cell surface hydrophobicity [4–6]. In some cases, cell surface components which help mediate adherence to hydrocarbons have been identified [4, 6–8].

One of the major limitations of this method is that quantitative criteria for adherence to hydrocarbons have not been well established; rather, semi-quantitative results, obtained by altering the oil: water ratio [2], or duration of the mixing procedure [3, 9], have usually been reported. In some cases, clear differences exist between bacteria which adhere in high proportions to hydrocarbons, and those which are nonadherent [2, 3]. However, among the many bacterial strains which do adhere, at least to some extent, comparison of their relative affinities is difficult. Thus, vague terms, such as 'pronounced', 'intermediate' or 'slight', have commonly been used to describe adherence to hydrocarbons. In order to use this method to compare the cell-surface hydrophobicity of various microbial cells in a more exact fashion, a quantitative criterion must be established to better describe their affinity for the oil: water interface. In the present communication, we offer such a criterion, based on the kinetics of adherence to hydrocarbon, as a function of hydrocarbon-to-water ratio.

Method

Provided that a steady state distribution of bacteria between the bulk aqueous phase and the oil:water interface can be established, the oil: water steady state distribution can serve as a criterion for bacterial hydrophobicity. Such a distribution would depend linearly on the oil: water volume ratio, the slope of such a dependence constituting the oil: water distribution coefficient of the bacterial cells. However, preliminary data indicated that this experimental approach would be problematic; mainly it is difficult to determine whether the extent of adherence, as a function of duration of mixing, reaches a plateau which represents a true steady state distribution. Moreover, for a steady state distribution to occur, binding of bacteria at the interface during the mixing ought to be reversible. Thus, increasing the water: oil ratio following the mixing procedure should result in release of cells into the aqueous phase. As we were unable to demonstrate such a phenomenon, an alternative quantitative approach, based on the rate, rather than extent, of adherence, was adopted.

Prior to testing the kinetics of adherence by turbidity measurements, two problems had to be addressed. First, when low hexadecane volumes are employed, it is conceivable that the droplets created by the mixing procedure possess a high specific gravity due to the adherence of large numbers of bacteria. This might prevent them from floating following mixing. Adherence experiments employing 10^9 bacteria (*Streptococcus pyogenes* M-5), 10 μl hexadecane and D_2O, rather than H_2O, revealed that the extent of decrease in turbidity following mixing was not affected by the increased specific gravity of the aqueous phase (not shown). This suggested that the flotation of oil droplets following mixing is not hampered by the adherence of bacteria, even at high bacteria: hydrocarbon ratios. Microscopic observation of the upper phase following the mixing demonstrated that the diameter of the cell-coated droplets was about the same (ca. 50 μm in diameter), irrespective of the hexadecane volume employed in our tests.

This observation helps explain the previous conclusion that all the hydrocarbon-associated bacteria float following the mixing procedure. It also assists in tackling a second potential problem, i.e., that the extremely low hexadecane volumes required might not provide sufficient surface area for adherence. The surface area of 1 μl (10^9 μm^3) hexadecane droplets of a radius of 25 μm is about 1.2×10^8 μm^2. Thus, the lowest hexadecane volume employed (10 μl) suffices to bind 1.2×10^9 bacteria of a diameter of 1 μm, if each cell occupies 1 μm^2 on the droplet surface. Therefore, all the experiments were carried out with less than 10^9 bacteria present in the assay.

The assay itself involves mixing of the bacterial suspension to be tested with a given volume of hydrocarbon for fixed, consecutive time periods. Following each agitation, the phases are allowed to separate, and the turbidity of the lower aqueous phase measured. We employed test tubes which can be read directly in the spectrophotometer without removal and transfer of the lower aqueous phase. Following measurement, the same mixture is then subjected to subsequent agitations.

Results and Discussion

The results of a typical experiment, using *Streptococcus pyogenes* M-5, previously reported to adhere to hexadecane [4, 11], are presented in Fig. 1. We observed that the decrease in turbidity as a function of time was exponential. Thus, linear results were obtained by plotting the logarithm of the percentage of cells remaining in the aqueous phase as a function of time. The slopes of the curves obtained (k) were observed to

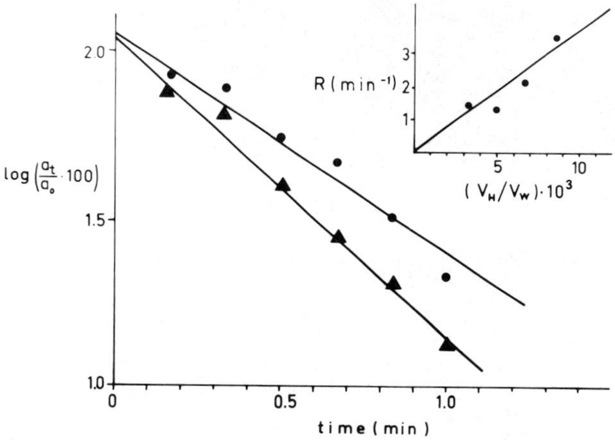

Fig. 1. The dependence of bacterial removal on the time of agitation. To 3 ml of bacterial suspension of an optical density (a_0) in the range of 0.35–0.40 as measured in a Junior Coleman, 10, 15, 20 or 25 μl of hexadecane were added. After each agitation period at a multivortex (SMI) setting of 2, the tubes were allowed to stand for 15 min for phase separation, and optical density of the aqueous phase in the same tube was then directly measured (a_t). Results obtained for 10 μl (●) and 20 μl (▲) are shown in the figure, in terms of the agitation time dependency of $\log(a_t/a_0) \cdot 100$ (results obtained for other volumes are excluded for sake of clarity). The insert describes the rate of removal (slopes of the curves obtained with different hexadecane volumes) as a function of the hexadecane: water ratio. Each determination represents the average of triplicate tubes. All curves showed linear regression with correlation coefficients in excess of 0.95. Bacteria were grown, washed and suspended in phosphate-buffered saline as previously reported [4].

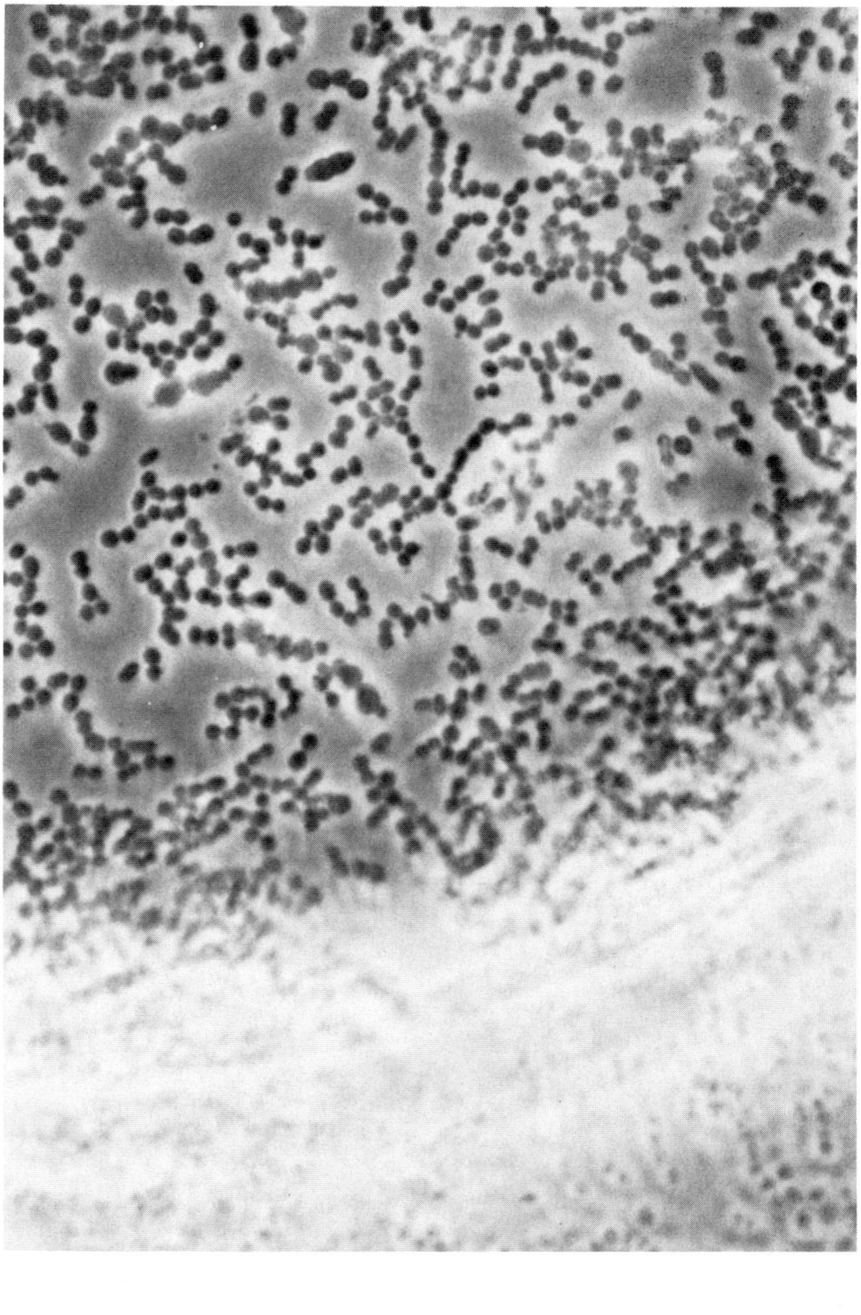

Fig. 2. Adhesion to hexadecane. Adhesion of *S. pyogenes* to large hexadecane droplet.

increase linearly with increasing hexadecane volume employed in the test. A similar kinetic approach has been employed by Kawabata et al. [11] in their studies on removal of bacteria from aqueous media by insoluble polymers. These authors studied the dependency of the rate constants of removal on the ratio of weight of polymer to the water volume and coined the term 'removal coefficient' for the slope of such dependencies. Thus, in our case, 'removal coefficient' refers to the change in the rate constant of removal of bacteria from the bulk aqueous phase to the hydrocarbon:water interface, as a function of the ratio of hydrocarbon to water in the assay. The insert in Fig. 1 presents the slopes obtained as a function of hydrocarbon: water ratio. We submit that the slope of the curve is a quantitative expression of the affinity of the cells tested for the water: oil interface, and denote it as the removal coefficient (K) of the cells from the bulk aqueous phase by the hydrocarbon. For *S. pyogenes*, K was 376 min^{-1}. Adherence of the cells to hexadecane droplets is shown in Fig. 2.

As Gibbons and Etherden pointed out [10], bacterial adherence to hydrocarbons yields reproducible results only when the conditions employed are carefully controlled (i.e., test tube dimensions). The kinetic approach suggested here should enable investigators to compare results obtained in their own laboratory, e.g., adherence of various strains, effects of growth conditions and treatments, in a quantitative fashion. Moreover, relative removal coefficients should be independent of experimental conditions as long as the identical conditions are employed to derive the removal coefficient.

Furthermore, in preliminary studies, we repeated the experiment shown in Fig. 1 by employing faster agitation while maintaining all other parameters identical. Although the rate constants were much higher than those described in Fig. 1, a similar removal coefficient (414 min^{-1}) was obtained. It is thus possible that the removal coefficient is independent of agitation conditions.

Preliminary experiments in our laboratories suggest the general applicability of the quantitative approach proposed in the present manuscript: (i) *S. pyogenes* cells, harvested from logarithmic growth phase, yielded results similar to those presented here for stationary phase cells, but with coefficients several orders of magnitude lower; (ii) other bacterial species (*Acinetobacter calcoaceticus*, *Serratia marcescens*) exhibit linear dependencies of the removal rate constants on the hexadecane: water ratio, enabling derivation of their removal coefficients, in the manner described here.

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References

1 Mudd, S. and Mudd, E. B. H. (1924) The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. 40, 633–645.

2 Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29–33.

3 Rosenberg, M. (1984) Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22, 289–295.

4 Ofek, I., Whitnack, E. and Beachey, E. H. (1983) Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. 154, 139–145.

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Bacterial Adherence at the Hydrocarbon–Water Interface

MEL ROSENBERG[1] and EUGENE ROSENBERG[2]

[1]School of Dental Medicine and Department of Human Microbiology,
Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel
[2]Department of Microbiology, George S. Wise Faculty of Life Sciences,
Tel-Aviv University, Ramat-Aviv Israel

Large differences were observed in the ability of eight *Acinetobacter calcoaceticus* strains to adhere to liquid hydrocarbon. Four strains exhibited extremely high affinity for hexadecane, two strains were moderately adherent and two other strains exhibited low affinity for the test hydrocarbon. Adherence of *A. calcoaceticus* RAG-1 to hexadecane was a function of culture age and growth conditions. In general, late exponential and stationary phase cells were more adherent than exponential phase cells. *A. calcoaceticus* RAG-1 cells cultured on hexadecane in flasks were adherent throughout growth. In contrast, a hydrocarbon-degrading strain of *Pseudomonas aeruginosa* adhered poorly to hydrocarbons, even following growth on hexadecane. Adherence of bacteria to hydrocarbons is discussed in terms of the bacterial cell surface, microbial growth on oil, and microbial contamination of surfaces.

1. INTRODUCTION

Bacterial adherence constitutes the first step in microbial colonization of interfaces (Marshall and Cruickshank, 1973; Dexter *et al.*, 1975; Marshall *et al.*, 1975; Dahlback *et al.*, 1981), often with detrimental results. Attachment of certain pathogenic microorganisms to the host tissue is a prerequisite for subsequent proliferation and disease (Alkan *et al.*, 1977; Beachey, 1980; Ofek *et al.*, 1982). The biofouling of submerged surfaces is initiated by layers of adherent bacteria (Dexter *et al.*, 1975; Characklis, 1981).

Adherence of bacteria to liquid hydrocarbons, a phenomenon often observed during petroleum fermentations (Marshall, 1976; Gutnick and Rosenberg, 1977; Neufeld *et al.*, 1980), has proven to be a general and useful tool in studying a variety of bacterial adherence phenomena. By means of a simple quantitative technique, large differences have been observed in the adherence of various bacterial species to liquid hydrocarbons (Rosenberg *et al.*, 1980). Correlations have been found between the ability of certain bacteria to adhere to hydrocarbons and their affinity for other surfaces, such as non-wettable plastic (Rosenberg, 1981), epithelial cells (Rosenberg *et al.*, 1981), and teeth (Weiss *et al.*, 1982).

Acinetobacter calcoaceticus RAG-1, originally isolated for growth on crude oil (Reisfeld *et al.*, 1972), adheres avidly to hydrocarbons during growth. A nonadherent mutant derived from RAG-1 was found to be unable to grow on hexadecane under conditions of low initial cell density and limited emulsification (Rosenberg and Rosenberg, 1981). In this paper, we present data on the effect of culture age and growth medium on the adherence of RAG-1 to hexadecane; adherence of

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Mel Rosenberg *is a Lecturer in Microbiology in the School of Dental Medicine and Department of Human Microbiology at Tel-Aviv University. His scientific interests deal with adherence and growth of micro-organisms on various surfaces.*
Eugene Rosenberg *is Professor of Microbiology at the Department of Microbiology, Tel-Aviv University. Scientific interests include microbial surfactants, oil microbiology and developmental biology of myxobacteria.*

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RAG-1 was also compared with that of seven other *Acinetobacter* strains, as well as a hydrocarbon-degrading strain of *Pseudomonas aeruginosa*. The data are discussed in terms of (i) specific cell-surface factors which may mediate or impede adherence to hydrocarbons, and (ii) the relative roles of adherence and emulsification in enabling bacterial growth on oil.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

Acinetobacter calcoaceticus RAG-1 (ATCC 31012), an oil-degrading bacterium, was previously isolated in this laboratory (Reisfeld *et al.*, 1972). Other *A. calcoaceticus* strains used in this study were provided by K. Bryn (Bryn *et al.*, 1977): B5W str 71 (ATCC 15149); B5W (ATCC 17903); ATCC 17906, CDC 7827 (ATCC 17959); ATCC 17977; ATCC 17985; strain '8' (*Bacterium anitratum* 8, State Serum Institute, Copenhagen, Denmark). *Pseudomonas aeruginosa* strain PAS279 was kindly provided by J. Shapiro. Strains were maintained on nutrient agar plates (Difco) supplemented with 0.5% NaCl; strains were maintained at 4°C and sub-cultured every two months.

2.2 MEDIA AND GROWTH CONDITIONS

Starter cultures were obtained by inoculating single colonies from plates into liquid medium. Erlenmeyer flasks were filled to 20% capacity with medium. Liquid cultures were incubated with shaking (New Brunswick G-53 gyratory shaker, New Brunswick, New Jersey) at 150 rev min^{-1} at 30°C. Experimental cultures were inoculated from starter cultures grown under the same conditions. The following liquid media were employed: NB—nutrient broth (Difco) supplemented with 0.5% NaCl; BHI—brain heart infusion broth (Difco); ethanol medium—PUM buffer (22.2 g $K_2HPO_4 . 3H_2O$; 7.26 KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 . 7H_2O$, and distilled water to 1000 ml, pH 7.1), supplemented with ethanol (0.5% v/v). *Pseudomonas* hexadecane medium—5 g NH_4NO_3, 2.5 g KH_2PO_4, 1 g $MgSO_4 . 7H_2O$, 50 mg threonine and tap water to 1000 ml, supplemented with 0.2% hexadecane; RAG-1 hexadecane medium—PUM buffer supplemented with hexadecane as indicated.

2.3 SOURCE OF MATERIALS

n-Hexadecane was obtained from Fluka, Switzerland (olefin-free, 99% purity); *n*-octane was synthetic grade (Merck, West Germany); *p*-xylene was obtained from Fluka (pure grade). All other chemicals and materials were of the highest grade available.

2.4 ADHERENCE TO LIQUID HYDROCARBONS

Bacteria were harvested from growth cultures by centrifugation at 8000 *g* for 10 min at 4°C, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm as indicated. Various volumes of the test hydrocarbons (*n*-hexadecane, *n*-octane or *p*-xylene) were added to round-bottom, acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed cell suspension. Following 10 min incubation at 30°C, the test tubes were uniformly vortexed on a Thermolyne Maxi Mix (Sybron Corp., Dubuque, Iowa) for 120 s. After allowing 15 min for the phases to separate, the bottom aqueous phase was carefully removed with a Pasteur pipette and transferred to disposable semi-micro cuvettes (C. A. Greiner and Sons, Nurtingen). Light absorbance of the lower aqueous phase was measured at 400 nm in a Gilford model 240 spectrophotometer, 1-cm light path, no filter. Adherence is expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure as compared to that of the cell suspension prior to the mixing procedure.

3. RESULTS

3.1 EFFECT OF GROWTH CONDITIONS ON ADHERENCE

The effect of different growth conditions on adherence of RAG-1 to hexadecane is summarized in Fig. 1. Adherence of cells grown in ethanol medium increased when the cells were harvested in stationary, rather than exponential, phase (Fig. 1A). When ethanol medium was supplemented with 0.05% casamino acids, exponential phase cells were completely nonadherent, whereas stationary phase cells adhered with similar affinity to the corresponding cells grown in the absence of

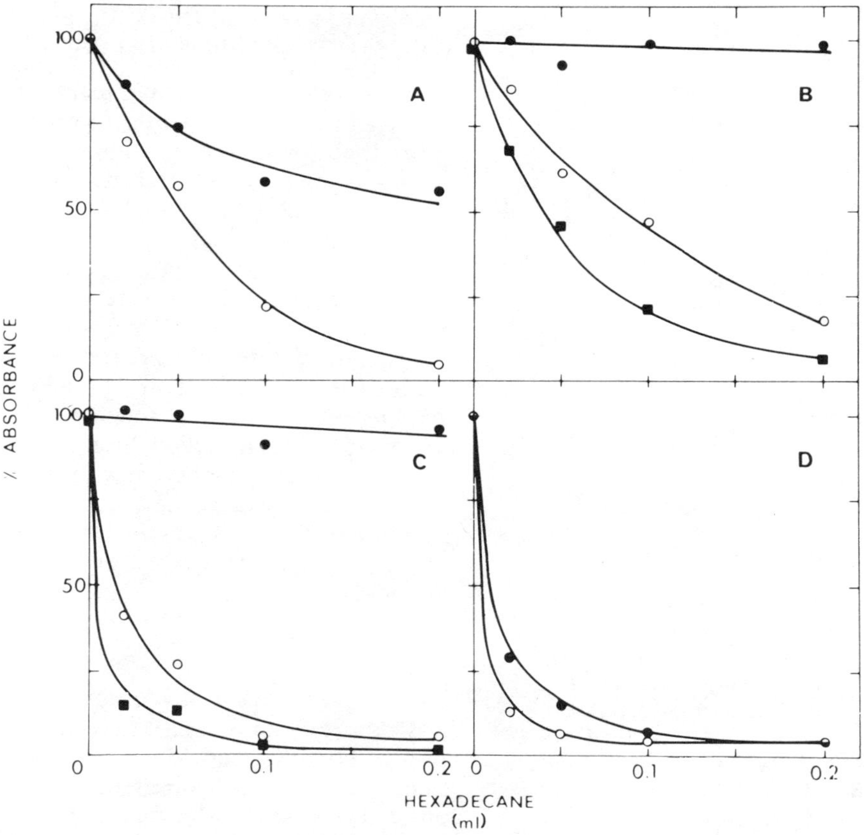

Fig. 1. Effect of culture age and medium composition on adherence of RAG-1 to hexadecane. Following growth as described below, RAG-1 cells were harvested, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4–1.6. Adherence to hexadecane was performed as described in Section 2.4. A, Exponential phase (●) and stationary phase (o) cells grown on minimal ethanol medium. B, Exponential phase (●), late exponential phase (o) and stationary phase (■) cells grown on minimal ethanol medium supplemented with 0.05% casamino acids. C, Exponential phase (●), late exponential phase (o) and stationary phase (■) cells grown in nutrient broth (NB) supplemented with 0.5% NaCl. D, Twenty-four-hour (●) and 54 h (o) cultures grown on 2% (v/v) hexadecane in shake flasks.

casamino acids (Fig. 1B). The greatest variations in adherence as a function of culture age were observed during growth on complex medium (Fig. 1C). Exponential phase cells exhibited negligible affinity for the test hydrocarbon; cells grown to late exponential or stationary phase, however, adhered with high affinity. As little as 0.02 ml hexadecane was able to bind 87% of RAG-1 cells grown to stationary phase on NB. RAG-1 cells grown on hexadecane medium in Erlenmeyer flasks remained extremely adherent throughout growth (Fig. 1D).

3.2 ADHERENCE OF ACINETOBACTER STRAINS

Adherence of eight strains of *Acinetobacter calcoaceticus* was compared following growth to stationary phase in BHI (Fig. 2). Adherence of RAG-1 cells grown on BHI was similar to that of NB-grown cells (Fig. 1C). Three other *Acinetobacter* strains also exhibited high affinity for the test hydrocarbon. Two strains (ATCC 17985 and ATCC 17906) were moderately adherent, and two strains (ATCC 17977 and 17903) exhibited low affinity towards hexadecane.

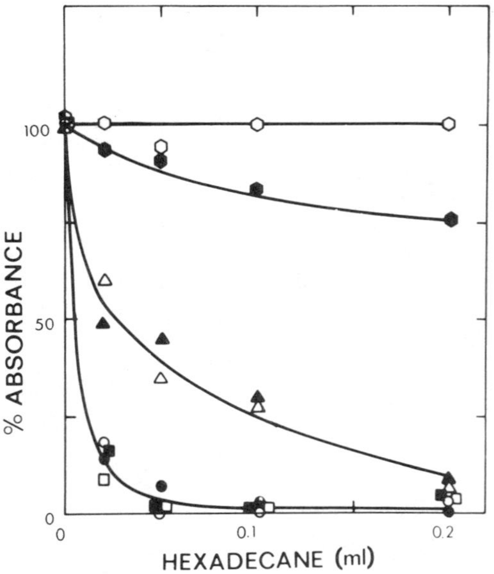

Fig. 2. Adherence of *Acinetobacter* strains to hexadecane. Cells were grown overnight to stationary phase in brain heart infusion broth, harvested, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4–1.6. Adherence to hexadecane was performed as described in Section 2.4. Symbols: (⬡), ATCC 17903; (⬢), ATCC 17977; (△), ATCC 17985; (▲), ATCC 17906; (o), RAG-1 (ATCC 31012); (■), ATCC 15149; (●), ATCC 17959; (□), Strain '8'.

3.3 COMPARISON OF THE ADHERENT PROPERTIES OF TWO HYDROCARBON-DEGRADING BACTERIA

In a separate series of experiments, adherence of RAG-1 to three test hydrocarbons was compared with that of another hydrocarbon-degrading, Gram-negative bacterium, *Pseudomonas aeruginosa* PAS279. Cells were examined following growth to late exponential phase on NB, and following 48 h growth on hexadecane in Erlenmeyer flasks. Results are presented in Fig. 3. Nutrient broth-grown *P. aeruginosa* cells exhibited no significant affinity towards any of the test hydrocarbons. The affinity of alkane-grown *P. aeruginosa* towards hexadecane did increase somewhat, following growth on this hydrocarbon, yet it remained nonadherent with respect to octane and xylene. In contrast, *A. calcoaceticus* RAG-1 cells adhered to all test hydrocarbons following growth on NB, and were even more adherent following growth on hexadecane.

4. DISCUSSION

Bacterial adherence at the aqueous–oil interface was reported almost sixty years ago in the classic studies of Mudd and Mudd (1924 *a*, *b*), and has since been frequently observed in bacterial growth on hydrocarbons (Marshall, 1976; Gutnick and

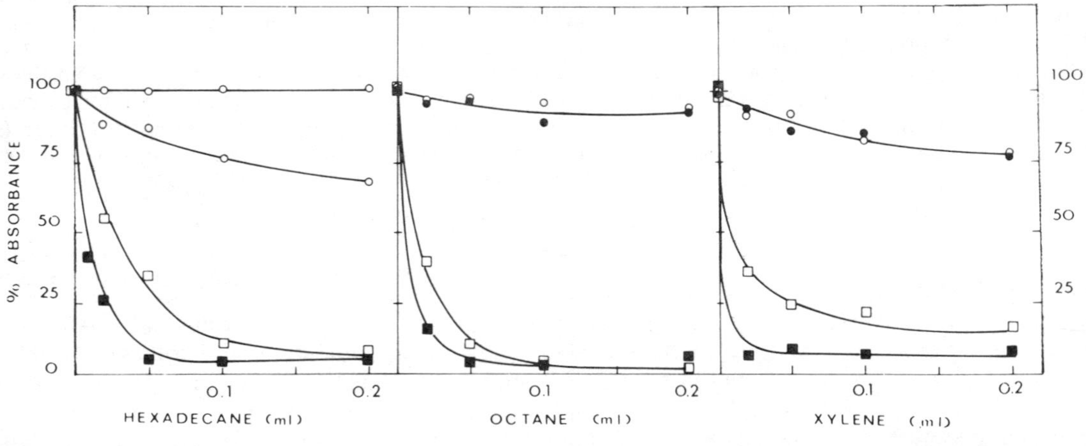

Fig. 3. Comparison of the adherence of two hydrocarbon-degrading strains, *A. calcoaceticus* RAG-1 and *Pseudomonas aeruginosa* PAS279. The cells were compared for adherence following growth to late exponential phase on nutrient broth, as well as following 48 h growth on hexadecane. Cells were washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.20–1.45. Adherence to the three test hydrocarbons was performed as described in Section 2.4. Symbols: (o), nutrient broth grown PAS279; (●) hexadecane grown PAS279; (□), nutrient broth grown RAG-1; (■), hexadecane (0.2%, v/v) grown RAG-1.

Rosenberg, 1977; Neufeld *et al.*, 1980). This phenomenon raises several interesting questions in petroleum microbiology: (i) What is the relationship between adherence to oil and growth of bacteria on oil? (ii) What cell surface components either promote or impede adherence of the bacterial cell to hydrocarbons and other hydrophobic surfaces? (iii) What is the relationship between partitioning of cells at the aqueous–hydrocarbon interface and adherence of the same cells to other surfaces? The development of a simple, quantitative technique for measuring bacterial adherence to hydrocarbons (Rosenberg *et al.*, 1980) has enabled us to address these and related questions.

In a previous report (Rosenberg *et al.*, 1980), as well as in the present study, it has been demonstrated that RAG-1 cells adhere with equivalent affinity to hydrocarbons which they do (i.e. hexadecane) or do not (i.e. octane, xylene) metabolize. Moreover, adherence to hydrocarbons is not restricted to hydrocarbon-degrading microorganisms; various bacteria which do not metabolize hydrocarbons, including strains of serratia, staphylococci, and streptococci, adhere avidly to hydrocarbons. The common denominator of these adherent strains is not growth on oil, but rather high cell surface hydrophobicity (Rosenberg *et al.*, 1980). These observations may lead one to assume that adherence to hydrocarbons and growth on oil are not related phenomena.

Furthermore, the poor affinity of *P. aeruginosa* PAS279 for hydrocarbons observed here appears to support such a premise. Nevertheless, experiments with RAG-1 have shown that adherence is, in fact, crucial for growth on hexadecane under conditions which do not allow emulsification of the hydrocarbon substrate (Rosenberg and Rosenberg, 1981). Nonadherent RAG-1 mutant MR-481 is only able to grow on hexadecane following the addition of emulsan, the potent extracellular emulsifying agent produced by RAG-1 cells (Rosenberg and Rosenberg, 1981). These experiments led to the conclusion that nonadherent strains may be able to grow on hydrocarbons in well-agitated closed systems, such as shake flasks and stirred fermentors which allow emulsification, or 'pseudosolubilization' (Chakravarty *et al.*, 1975) of the substrate.

However, such nonadherent strains would not grow on poorly agitated hydrocarbons as would occur in most environmental situations, such as water-containing oil tanks, oil deposits, or oil spills. In poorly agitated systems, the concentration of hydrocarbon in the bulk aqueous phase would hardly be sufficient to enable growth. Similarly, in the open environment, emulsified oil would tend to diffuse away from nonadherent cells. Thus, while nonadherent strains can be grown on oil under laboratory conditions, adherence is an important property for hydrocarbon-degrading microorganisms in the open environment (Fig. 4.). This point has been largely ignored in research concerned with hydrocarbon-degrading *Pseudomonas* strains intended for inoculation into oil spills, as even bacteria with high levels of hydrocarbon-oxidizing enzymes may not proliferate unless they are able to adhere to the bulk hydrocarbon substrate (Gutnick and Rosenberg, 1977; Rosenberg and Rosenberg, 1981). This problem can be overcome by the initial use of an adherent microorganism which can then be subjected to genetic manipulations; a *P. aeruginosa* strain which adheres with very high affinity for hydrocarbons, and is capable of growing on hexadecane, has recently been identified (M. Rosenberg and T. Meshulam, unpublished data).

In this paper, large variations were observed in the adherent properties of RAG-1 as a function of growth conditions. These results can be explained by the expression of specific cell-surface components which may promote, or impede, adherence to hydrocarbons. In a previous study we provided evidence for the role of thin fimbriae in adherence of RAG-1 to hydrocarbons and to polystyrene (Rosenberg *et al.*, 1982). The poor affinity of exponential phase cells harvested from complex medium may be due to the lack of thin fimbriae in such cells, whereas cells harvested from latter stages of growth are increasingly fimbriate (M. Rosenberg and E. A. Bayer, unpublished data).

At least one surface component of RAG-1 impedes its adherence to hydrocarbons: emulsan, which is present on the cell surface prior to its release, lowers the affinity of RAG-1 for hydrocarbon (Rosenberg *et al.*, 1983*a*). Following enzymatic degradation of cell-associated emulsan, adherence was found to increase (Rosenberg *et al.*, 1983*b*). Similarly, emulsan-deficient mutants adhered with higher affinity than corresponding

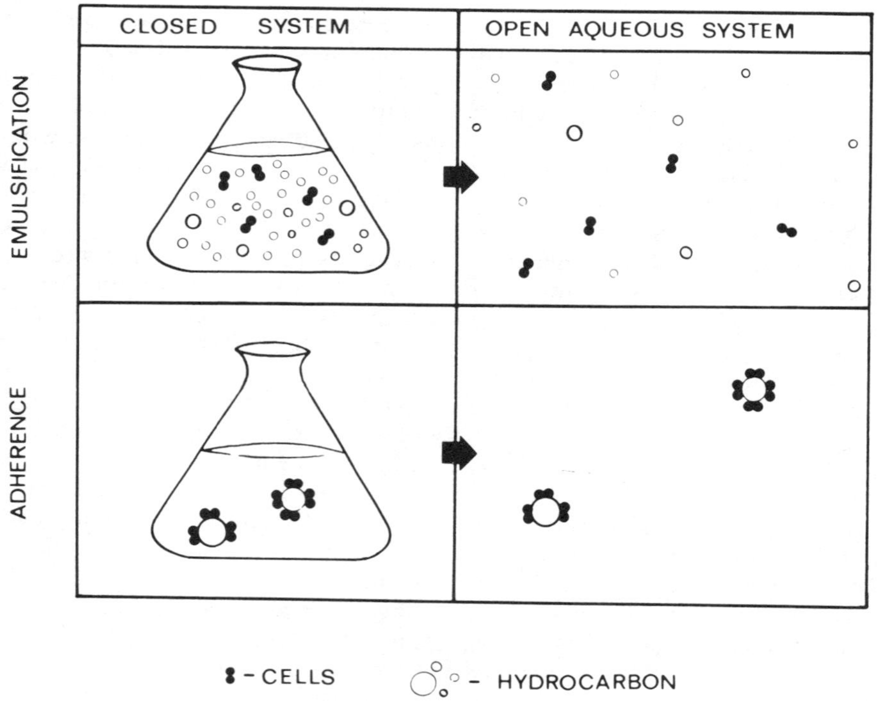

Fig. 4. Model: Adherence versus emulsification in microbial growth on liquid hydrocarbons. While the availability of emulsified hydrocarbon in a closed system (e.g. fermentor or shake flask) may enable growth of nonadherent cells (top left), the introduction of such a mixture into an open aqueous environment would result in diffusion of the dispersed hydrocarbon away from the cells, thereby lowering substrate availability and restricting growth. Adherent cells, transferred from a closed (bottom left) to an open aqueous environment (bottom right), would remain in direct contact with the growth substrate, enabling growth.

wild type RAG-1 cells. Since RAG-1 cells release emulsan from the cell surface into the medium during the latter stages of growth (Rubinowitz *et al.*, 1982), it is likely that the increased affinity of stationary phase ethanol-grown cells towards hexadecane is due to the relative absence of cell-bound emulsan. Of interest in this context is the observation that extracellular emulsan is similarly able to prevent adherence of RAG-1 cells to hydrocarbon (Rosenberg *et al.*, 1983*a*).

These results emphasize the importance of growth conditions in determination of the adherent properties, or, alternatively, the cell-surface hydrophobicity of bacterial cells.

The large variations observed in the adherence of various *Acinetobacter* strains to hexadecane show that, for a given set of growth conditions, adherence to hydrocarbons may be strain specific. This does not necessarily imply that the poorly adherent strains lack cell-surface components which can mediate adherence to hydrocarbons. A heavily encapsulated strain of *A. calcoaceticus*, BD4, does not adhere to hexadecane at all; however, following enzymatic treatment which removes the capsule, the cells become extremely adherent (Rosenberg *et al.*, 1983*b*). Thus, in at least one *A. calcoaceticus* strain, adherence to hexadecane can be completely masked by the presence of capsular polysaccharide.

In summary, it has been shown that adherence may be a central factor in bacterial growth on oil in the environment. The data demonstrate that adherent properties may vary widely among different oil-degrading species, and even among strains of a single species. Moreover, adherence is clearly related to growth conditions. These parameters should be taken into consideration when inoculation of bacteria into the environment, for purposes of oil degradation, is contemplated. Bacterial adherence to oil may illustrate a general microbial phenomenon: a biological advantage is conferred upon microorganisms which can escape from the bulk aqueous phase to an interface which provides nutrition and enables growth.

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REFERENCES

Alkan, M., Ofek, I. and Beachey, E. H. 1977. Adherence of pharyngeal and skin strains of group A *Streptococci* to human skin and oral epithelial cells. *Infect. Immun.* **18**, 555–557.

Beachey, E. H. (ed.) 1980. *Bacterial Adherence*, Chapman and Hall, London and New York.

Bryn, K., Jantzen, E. and Bovre, K. 1977. Occurrence and patterns of waxes in Neisseriaceae. *J. Gen. Microbiol.* **102**, 33–43.

Chakravarty, M., Singh, H. D. and Baruah, J. N. 1975. A kinetic model for microbial growth on liquid hydrocarbons. *Biotechnol. Bioeng.* **17**, 399–412.

Characklis, W. G. 1981. Fouling biofilm development: A process analysis. *Biotechnol. Bioeng.* **23**, 1923–1960.

Dahlback, B., Hermansson, M., Kjelleberg, S. and Norkrans, B. 1981. The hydrophobicity of bacteria—an important factor in their initial adhesion at the air–water interface. *Arch. Microbiol.* **128**, 267–270.

Dexter, S. C., Sullivan, J. D., Jr, Williams, J. III and Watson, S. W. 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. *Appl. Microbiol.* **30**, 298–308.

Gutnick, D. L. and Rosenberg, E. 1977. Oil tankers and pollution: a microbiological approach. *Ann. Rev. Microbiol.* **31**, 379–396.

Marshall, K. C. 1976. *Interfaces in Microbial Ecology*, Harvard University Press, Cambridge, Massachusetts.

Marshall, K. C. and Cruickshank, R. H. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. *Arch. Mikrobiol.* **91**, 29–40.

Marshall, K. C., Cruickshank, R. H. and Bushby, H. V. A. 1975. The orientation of certain root-nodule bacteria at interfaces, including legume root-hair surfaces. *J. Gen. Microbiol.* **91**, 198–200.

Mudd, S. and Mudd, E. B. H. 1924*a*. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. *J. Exp. Med.* **40**, 633–645.

Mudd, S. and Mudd, E. B. H. 1924*b*. Certain interfacial tension relations and the behaviour of bacteria in films. *J. Exp. Med.* **40**, 647–660.

Neufeld, R. J., Zajic, J. E. and Gerson, D. F. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. *Appl. Environ. Microbiol.* **39**, 511–517.

Ofek, I., Simpson, W. A. and Beachey, E. H. 1982. The formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. *J. Bacteriol.* **149**, 426–433.

Reisfeld, A., Rosenberg, E. and Gutnick, D. 1972. Microbial degradation of crude oils: factors affecting the dispersion in sea water by mixed and pure cultures. *Appl. Microbiol.* **24**, 363–368.

Rosenberg, E., Gottlieb, A. and Rosenberg, M. 1983*a*. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. *Infect. Immun.* **39**, 1024–1028.

Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M. and Gutnick, D. L. 1983*b*. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hexadecane. *FEMS Microbiol. Lett.* **17**, 157–160.

Rosenberg, M. 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Appl. Environ. Microbiol.* **42**, 375–377.

Rosenberg, M. and Rosenberg, E. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane. *J. Bacteriol.* **148**, 51–57.

Rosenberg, M., Gutnick, D. and Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* **9**, 29–33.

Rosenberg, M., Perry, A., Bayer, E. A., Gutnick, D. L., Rosenberg, E. and Ofek, I. 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. *Infect. Immun.* **33**, 29–33.

Rosenberg, M., Bayer, E. A., Delarea, J. and Rosenberg, E. 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. *Appl. Environ. Microbiol.* **44**, 929–937.

Rubinowitz, C., Gutnick, D. L. and Rosenberg, E. 1982. Emulsan production by *Acinetobacter calcoaceticus* RAG-92 in the presence of chloramphenicol. *J. Bacteriol.* **152**, 126–132.

Weiss, E., Rosenberg, M., Judes, H. and Rosenberg, E. 1982. Cell surface hydrophobicity of adherent oral bacteria. *Current Microbiol.* **7**, 125–128.

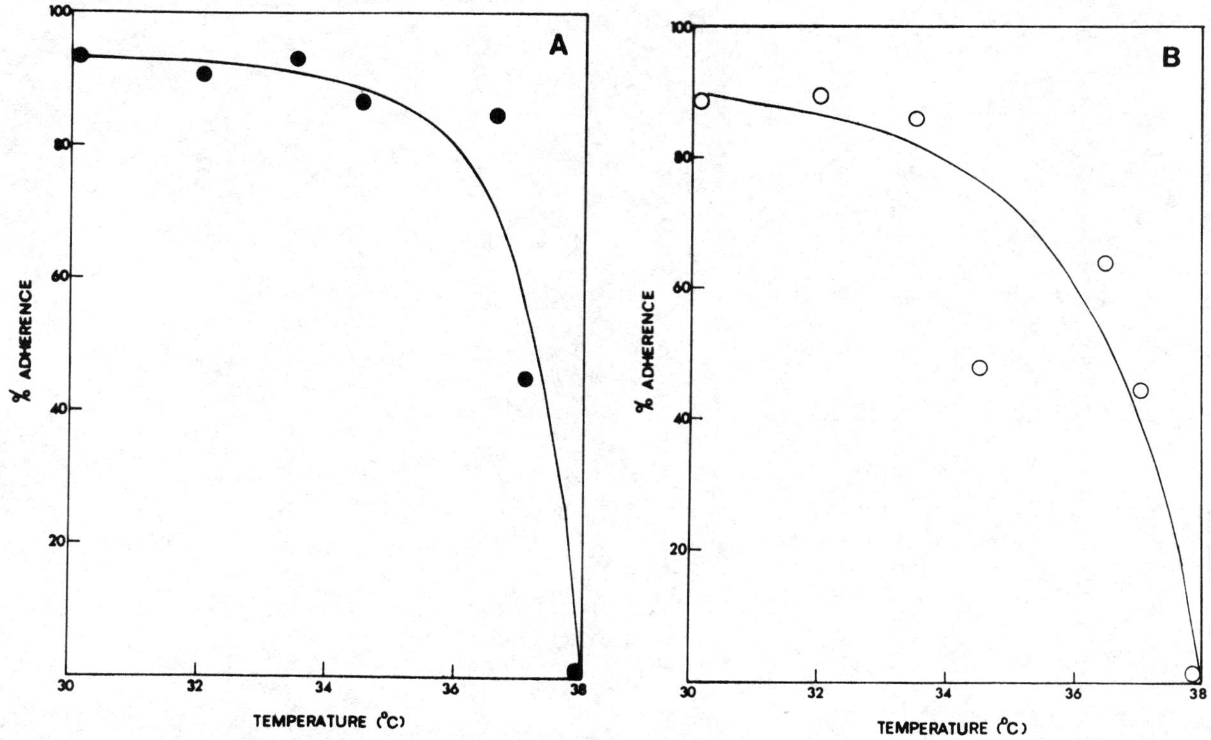

FIG. 1. Effect of growth temperature on adherence to hexadecane. Cells were grown at various temperatures and tested for adherence to 0.2 ml of hexadecane as described in Materials and Methods. The results are presented as the percent adherence of pigmented strain 1785 (A) and nonpigmented strain 3255 (B) to hexadecane as a function of growth temperature. Similar results were obtained for pigmented strain RZ (data not shown).

100-μl portions of a bacterial suspension were placed on polystyrene petri dishes (Miniplast, Ein Shemer, Israel), and following 30 min of incubation at room temperature, each surface was washed vigorously with tap water (21). Adherence was determined visually following staining with Gentian violet (21). The presence of mannose-sensitive adhesins was tested by examining the aggregation of yeast cells, essentially as previously described (15, 16). Dried cells of *Saccharomyces cerevisiae* (Fleischmann's active dry yeast) were suspended in PUM buffer to a concentration of 5 mg/ml. Equal volumes (20 μl) of bacterial and yeast suspensions were mixed on glass slides, and aggregation was scored visually. To ensure that the phenomenon was mannose inhibitable, the assay was simultaneously carried out in the presence of 0.2% (final concentration) D-mannose as a control.

TABLE 1. Adherence characteristics of pigmented *Serratia* strains grown at 30°C

Strain	% Adherence to:[a]			Adherence to polystyrene[b]	Mannose-sensitive aggregation of yeast cells[c]
	n-Hexadecane	*n*-Octane	*p*-Xylene		
183	92	91	90	+	+
55	91	93	88	+	–
1785	94	94	91	+	+
SH-1	78	80	74	+	+
RZ	96	96	95	+	+
1704	84	80	72	+	+
1515	94	85	88	+	+
II_9A	89	90	82	+	–
BY-1	92	90	80	+	–
I_5	84	85	68	+	–
Mean	89	88	83		

[a] Experiments to determine adherence to hydrocarbons were performed as described in Materials and Methods. The results (means of two separate experiments) are expressed as percent decreases in turbidity compared with the initial cell suspension.

[b] Adherence to polystyrene was scored visually following staining with Gentian violet as described in Materials and Methods.

[c] Aggregation of yeast cells was scored visually as described in Materials and Methods.

RESULTS

Figure 1 shows the effect of growth temperature on the adherence of a pigmented strain and a nonpigmented strain to the test hydrocarbon hexadecane. Both strains were highly adherent following growth at 30°C but became nonadherent as the growth temperature approached 38°C. Of

TABLE 2. Adherence characteristics of nonpigmented *Serratia* strains grown at 30°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
3255	88	90	88	+	–
577	59	62	62	+	+
2861	62	57	55	+	+
BY-2	80	88	76	+	–
Mean	72	74	70		

[a] For experimental conditions see the footnotes to Table 1.

TABLE 3. Adherence characteristics of pigmented *Serratia* strains grown at 38°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
183	0	0	0	−	+
55	0	0	0	−	−
1785	0	1.1	13	−	+
SH-1	0	0	0	−	+
RZ	2.9	2.2	2.3	−	+
1704	0.8	1.4	8.3	−	+
1515	0	0	0.5	−	+
II$_b$A	0	0	3.3	−	−
BY-1	3.4	0.3	1.1	−	−
I$_5$	3.0	3.5	4.0	−	−
Mean	1.0	0.9	3.3		

[a] For experimental conditions (except the growth temperature of cells) see the footnotes to Table 1.

considerable interest was the observation that adherence in the pigmented strain did not correlate directly with pigment synthesis; whereas strain 1785 did not produce pigment when it was grown at temperatures above 36°C, a high proportion (84%) of the cells grown at 36.5°C adhered to hexadecane.

The ability of both pigmented and nonpigmented *Serratia marcescens* strains to adhere to hydrocarbons when they were grown at 30°C is summarized in Tables 1 and 2. The mean level of adherence of the pigmented strains to all three test hydrocarbons (hexadecane, octane, and xylene) (Table 1) was about 15% higher than the level observed for the nonpigmented strains (Table 2). Similarly, all strains grown at 30°C adhered to the hydrophobic solid polystyrene (Tables 1 and 2). In contrast, neither pigmented nor nonpigmented strains adhered to hydrocarbons or to polystyrene when they were grown at 38°C (Tables 3 and 4).

Tables 1 through 4 show the ability of pigmented and nonpigmented isolates to exhibit mannose-sensitive aggregation of yeast cells following growth at 30 or 38°C.

DISCUSSION

The ability of microorganisms to adhere to solid surfaces and to partition at liquid-air and liquid-liquid interfaces is currently regarded as an important factor in bacterial colonization and survival in various habitats (24a). One of the major factors in promoting adherence both in open environments and in hosts is bacterial hydrophobicity. In this context, *Serratia* strains are of considerable interest for the following reasons: (i) they are colonizers of interfaces in the open environment and have also been increasingly implicated in a wide variety of infections in humans (26); and (ii) the avid hydrophobicity observed in pigmented *Serratia* cells has been correlated with their ability to concentrate at air-water interfaces (2–6, 8, 9, 12–14, 28) and water-oil interfaces (22, 24, 24a) and to adhere to solid surfaces (22, 24). Whereas considerable attention has been given to the potential role of *Serratia* hydrophobicity in enabling the colonization of the sea surface by these organisms (2, 6, 9, 14, 28), their dispersion in aerosols and jet bubbles (3–5), and their nutrient scavenging on solid surfaces (12), the possible role of surface hydrophobicity in affecting the infective nature of *Serratia* strains has not been studied.

TABLE 4. Adherence characteristics of nonpigmented *Serratia* strains grown at 38°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
3255	0	0	0	−	−
577	0	0	5.9	−	+
2861	0	0	2.1	−	+
BY-2	6.2	0	6.4	−	−
Mean	1.6	0	3.6		

[a] For experimental conditions (except the growth temperature) see the footnotes to Table 1.

Our results suggest that most or all clinical *Serratia marcescens* isolates are capable of exhibiting pronounced cell surface hydrophobicity, as determined by adherence to hydrocarbons and polystyrene.

Infectious strains of *Serratia marcescens* may be either pigmented or nonpigmented, with the latter type predominating (7). Since the presence of the pigment prodigiosin has been cited as the factor which is responsible for hydrophobicity in *Serratia* strains (3, 8, 13, 21, 24), we investigated the hydrophobic properties of both pigmented and nonpigmented strains under growth conditions which either allowed or inhibited prodigiosin synthesis. Williams et al. (30, 31) have reported that prodigiosin synthesis occurs at 30°C, but is completely inhibited by growth at higher temperatures. In order to conveniently monitor pigmentation, cells were grown on solid media.

The finding that the mean level of adherence of the pigmented strains was somewhat higher than that of the nonpigmented strains when the organisms were grown at 30°C suggests that the pigment may contribute somewhat to cell surface hydrophobicity. However, the following two lines of evidence indicate that another component(s) is involved: (i) the adherence of all four nonpigmented strains to the test hydrocarbons and to polystyrene following growth at 30°C, and (ii) the observation that strain 1785 remained hydrophobic following growth at 36.5°C, even though pigmentation was no longer visually apparent. These results are consistent with a recent report that a nonpigmented mutant of strain RZ retained partial ability to adhere to hydrocarbons and polystyrene (22).

Preliminary results (data not shown) have shown that a major outer surface protein (molecular weight, approximately 95,000) can be released by shearing (in the presence of 0.2% sodium dodecyl sulfate) from the surfaces of pigmented and nonpigmented strains grown at 30°C, but is absent from the same cells when they are grown at 38°C. The possible contribution of this protein to the cell surface hydrophobicity of pigmented and nonpigmented strains is under investigation.

Several studies on mannose-sensitive adhesins in *E. coli* have suggested that the presence of these adhesins increases cell surface hydrophobicity (11, 27). Since most clinical and other isolates of *Serratia* bear mannose-sensitive adhesins (1, 15, 18), it was of interest to compare cell surface hydrophobicity with mannose-sensitive aggregation of yeast cells. We found no evidence that the ability of clinical *Serratia* isolates to aggregate yeast cells in a mannose-inhibitable fashion is related to the observed hydrophobicity: (i) among the strains that exhibited pronounced cell surface hydrophobicity were strains which did (e.g., strains 183 and

1785 [Table 1]) and did not (e.g., strains 55 and BY-1 [Table 1]) aggregate yeast cells; and (ii) strains which were mannose sensitive following growth at 30°C (Tables 1 and 3) exhibited the same characteristic following growth at 38°C (Tables 2 and 4) despite their complete loss of hydrophobic surface properties following growth at the higher temperature. A separate study has similarly suggested that cell surface hydrophobicity and mannose-specific adherence in *Serratia marcescens* are mutually independent; adherence to hydrocarbons was inhibited by the amphipathic polymer emulsan (20) but not by mannose, whereas adherence of the same bacteria to yeast cells was inhibited by mannose but not by emulsan (M. Rosenberg, A. Gottlieb, and E. Rosenberg, unpublished data).

The finding that all of the clinical isolates tested were hydrophobic following growth at 30°C but lost this property at higher temperatures is of interest. Ofek et al. (17) have implied that pathogenic *Streptococcus pyogenes* cells may regulate their cell surface hydrophobicity, presenting a hydrophobic outer surface in order to promote initial attachment to host tissue, but at some later stage exposing a hydrophilic surface so as to evade phagocytosis (29). Since infections mediated by *Serratia marcescens* often originate in bacteria that are present in the immediate surroundings (26), in which the cells are usually at lower-than-body temperature, the *Serratia marcescens* cells may conceivably be hydrophobic during initial contact with host tissue or with plastic surfaces in contact with a host (10) but not hydrophobic following the onset of infection.

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LITERATURE CITED

1. **Adegbola, R. A., and D. C. Old.** 1982. New fimbrial hemagglutinin in *Serratia* species. Infect. Immun. **38:**306–315.
2. **Bezdek, H. F., and A. F. Carlucci.** 1972. Surface concentration of marine bacteria. Limnol. Oceanogr. **17:**566–569.
3. **Blanchard, D. C., and L. D. Syzdek.** 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. **23:**389–400.
4. **Blanchard, D. C., and L. D. Syzdek.** 1982. Water-to-air transfer and enrichment of bacteria in drops from bursting bubbles. Appl. Environ. Microbiol. **43:**1001–1005.
5. **Blanchard, D. C., L. D. Syzdek, and M. E. Weber.** 1981. Bubble scavenging of bacteria in freshwater quickly produces bacterial enrichment in airborne jet drops. Limnol. Oceanogr. **26:** 961–964.
6. **Carlucci, A. F., and P. M. Williams.** 1965. Concentration of bacteria from sea water by bubble scavenging. J. Cons. Perm. Int. Explor. Mer **30:**28–33.
7. **Ding, M.-J., and R. P. Williams.** 1983. Biosynthesis of prodigiosin by white strains of *Serratia marcescens* isolated from patients. J. Clin. Microbiol. **17:**476–480.
8. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T.-A. Stenstrom.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesin to air-water interfaces. Arch. Microbiol. **131:**308–312.
9. **Hermansson, M., S. Kjelleberg, and B. Norkrans.** 1979. Interaction of pigmented wild type and pigmentless mutant of *Serratia marcescens* with lipid surface film. FEMS Microbiol. Lett. **6:**129–132.
10. **Hogt, A. H., J. Dankert, and J. Feijen.** 1983. Encapsulation, slime production and surface hydrophobicity of coagulase-negative staphylococci. FEMS Microbiol. Lett. **18:**211–215.
11. **Jann, K., G. Schmidt, E. Blumenstock, and K. Vosbeck.** 1981. *Escherichia coli* adhesion to *Saccharomyces cerevisiae* and mammalian cells: role of piliation and surface hydrophobicity. Infect. Immun. **32:**484–489.
12. **Kefford, B., S. Kjelleberg, and K. C. Marshall.** 1982. Bacterial scavenging: utilization of fatty acids localized at a solid-liquid interface. Arch. Microbiol. **133:**257–260.
13. **Kjelleberg, S., C. Lagercrantz, and T. Larsson.** 1980. Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. FEMS Microbiol. Lett. **7:**41–44.
14. **Kjelleberg, S., and T. A. Stenstrom.** 1980. Lipid surface films: interaction of bacteria with free fatty acids and phospholipids at the air/water interface. J. Gen. Microbiol. **116:**417–423.
15. **Mirelman, D., G. Altmann, and Y. Eshdat.** 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. J. Clin. Microbiol. **11:**328–331.
16. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. Infect. Immun. **22:**247–254.
17. **Ofek, I., E. Whitnack, and E. H. Beachey.** 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. **154:**139–145.
18. **Old, D. C., R. Adegbola, and S. S. Scott.** 1983. Multiple fimbrial haemagglutinins in *Serratia* species. Med. Microbiol. Immunol. **172:**107–115.
19. **Purkayastha, M., and R. P. Williams.** 1960. Association of pigment with the cell envelope of *Serratia marcescens* (*Chromobacterium prodigiosum*). Nature (London) **187:**349– 351.
20. **Rosenberg, E., A. Gottlieb, and M. Rosenberg.** 1983. Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. Infect. Immun. **39:**1024–1028.
21. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42:**375–377.
22. **Rosenberg, M.** 1984. Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. J. Bacteriol. **160:**480–482.
23. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. Appl. Environ. Microbiol. **44:**929–937.
24. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:**29–33.

24a. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. Adv. Microbial Ecol. **9:**353–393.

25. **Rosenberg, M., E. Rosenberg, H. Judes, and E. Weiss.** 1983. Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. FEMS Microbiol. Lett. **20:**1–5.
26. **Sleigh, J. D.** 1983. Antibiotic resistance in *Serratia marcescens*. Br. Med. J. **287:**1651–1653.
27. **Smyth, C. J., P. Jonsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjerten, and T. Wadstrom.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22:**462–472.
28. **Syzdek, L. D.** 1982. Concentration of *Serratia* in the surface microlayer. Limnol. Oceanogr. **26:**961–964.
29. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32:**19–40.
30. **Williams, R. P.** 1973. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. Appl. Microbiol. **116:** 259–268.
31. **Williams, R. P., C. L. Gott, S. M. H. Qadri, and R. H. Scott.** 1971. Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. J. Bacteriol. **106:**438–443.

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Saliva-mediated inhibition of adhesion of hydrophobic oral and non-oral bacteria to polystyrene

M. Rosenberg, N. Regimov and I. Eli, Tel Aviv

Various investigations in recent years have underlined the importance of hydrophobic interactions in adhesion of bacteria to oral surfaces. In the present investigation, the possible role of saliva in affecting adhesion mediated by hydrophobic interactions was studied in a simple and reliable in vitro system, based on bacterial adhesion to polystyrene microtiter plates. Saliva inhibited adhesion of both oral (Streptococcus mutans) and non-oral bacteria (Serratia marcescens) in a dose-dependent fashion; inhibition of over 90% was observed at high saliva concentrations. Of considerable interest was the finding that mixing of saliva in the presence of the liquid hydrocarbon, hexadecane, abolished the inhibitory ability, presumably due to extraction of the inhibitory components to the oil:water interface. The inhibitory effect of the saliva could be restored by elution of such components back into the bulk aqueous phase. Among the hexadecane-extracted and eluted salivary components, several proteins were found. Their possible role in inhibition of bacterial adhesion to hydrophobic surface is currently under study.

(22)

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Hydrophobic Interactions: Role in Bacterial Adhesion

MEL ROSENBERG and STAFFAN KJELLEBERG

1. Introduction

The last decade has seen a dramatic rise in scientific interest in the field of bacterial adhesion and related subjects. Much of this interest has been directed in search of "specific interactions" between bacterium and substratum, such as those that are inhibited by specific sugar moieties. A second avenue of research has been the study of the role of less specific, hydrophobic interactions. Since it appears that this second avenue of research is being increasingly followed, reviews dealing with bacterial hydrophobicity and adhesion should be of use. The aims of the present chapter are to (1) present some relevant aspects of hydrophobic interactions; (2) describe the methodology available for measurements related to bacterial cell-surface hydrophobicity and the parameters they may measure; (3) discuss investigations dealing with surface components that promote or reduce bacterial hydrophobicity; and (4) survey studies related to the role of hydrophobic interactions in mediating bacterial adhesion to interfaces, traversing areas of environmental and medical interest.

The importance of bacterial hydrophobicity in adhesion to liquid–liquid interfaces and the possible relationship between cell-surface hydro-

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MEL ROSENBERG • School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel. **STAFFAN KJELLEBERG** • Department of Marine Microbiology, University of Göteborg, S-413 19 Göteborg, Sweden.

phobicity and phagocytosis were reported over 60 years ago by Mudd and Mudd (1924a,b). These authors observed that certain microorganisms (e.g., *Erythrobacillus prodigiosus,* currently known as *Serratia marcescens*) tend to partition at the water–oil interface, and provided a theoretical definition of this phenomenon in terms of bacteria–oil, bacteria–water, and oil–water interfacial energies. Reed and Rice (1931) made an unsuccessful attempt to quantify bacterial partitioning at the oil–water interface, presumably because of their limited choice of bacterial strains. However, these authors were able to show that certain bacteria (e.g., mycobacteria) are able to pass from the bulk aqueous phase to the bulk oil phase upon vigorous mixing.

In the subsequent four decades, relatively little scientific interest was directed toward the study of bacterial hydrophobicity, with notable exceptions in the work of Dyar (1948) and Hill *et al.* (1963) and studies on bacterial interactions at the air–water interface (e.g., Boyles and Lincoln, 1958). Interest in bacterial hydrophobicity and adhesion was renewed with the work of Marshall and coworkers (Marshall and Cruickshank, 1973; Marshall *et al.,* 1975) and van Oss and colleagues [for a review see van Oss (1978)] in the early 1970s.

During the past decade, a variety of techniques for measuring bacterial hydrophobicity have been proposed, accompanied by a surge in research, as can be discerned from Fig. 1.

Hydrophobic interactions have been implicated in a wide array of adhesion phenomena, including partitioning at the oil–water (M. Rosenberg, 1984a) and air–water (Kjelleberg, 1985) interfaces and adhesion to plastics (Fletcher, 1976; Fletcher and Marshall, 1982b), epithelial cells (M. Rosenberg *et al.,* 1981; E. Rosenberg, *et al.,* 1983a), phagocytes (van Oss, 1978), teeth (M. Rosenberg *et al.,* 1983a,c; Nesbitt *et al.,* 1982), sub-

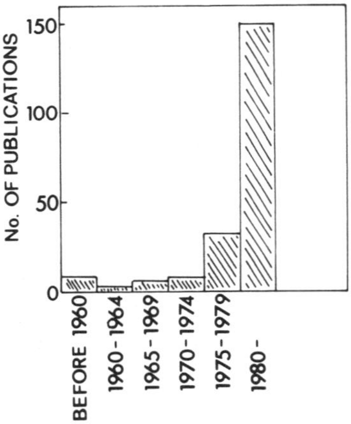

Figure 1. Hydrophobicity- and adhesion-related papers as a function of publication period. As an indication of interest in hydrophobicity and adhesion, the distribution of the publications referred to in this review into publication periods is illustrated. A burst in interest over the past decade is evident.

merged aquatic surfaces (Fattom and Shilo, 1984), sulfur granules (Bryant *et al.,* 1984), mineral surfaces (Stenstrom and Kjelleberg, 1985), and to one another (Stanley and Rose, 1967; Moore and Marshall, 1981; Faris *et al.,* 1983). In several cases, surface components contributing to bacterial hydrophobicity have been identified. Bacterial hydrophobicity may also be an important factor in other adhesion-related phenomena, such as gliding motility (Dworkin *et al.,* 1983; Humphrey *et al.,* 1979; R. Wolkin and J. L. Pate, in preparation) and colony spreading (Gibbons *et al.,* 1983).

Despite the apparent importance of hydrophobic interactions in participating in such a wide range of phenomena, the term "bacterial hydrophobicity" has no unequivocal definition, nor a definitive scale of values. This is not surprising in light of current disagreements among scientists on the precise nature of hydrophobic interactions (e.g., Hildebrand, 1979; Israelachvili and Pashley, 1984) and the lack of consensus on a definitive method for measuring bacterial hydrophobicity (see Section 3). Thus, the literature is replete with confusing terminologies ("pronounced" versus "slight" cell-surface hydrophobicity) and results from various laboratories that cannot be compared with one another.

Are we then to conclude that the plurarity of methods for measuring bacterial hydrophobicity is detrimental? Actually, the opposite is probably the case. The nonuniformity of the bacterial cell surface confounds attempts at a universal criterion: a bacterium that is hydrophilic all over, save for a hydrophobic tip (Marshall and Cruickshank, 1973), may be hydrophilic in one assay and hydrophobic in another. Moreover, the various techniques have given researchers flexibility in approaching various adhesion problems.

Cell-surface hydrophobicity is by no means a subject of interest restricted to bacteriology, and has been implicated in a wide variety of eucaryotic cell interactions and viral adhesion as well. Although such phenomena are beyond the scope of this review, we have listed some examples in Table I.

2. The Hydrophobic Effect

The general concept of hydrophobic interactions in adhesion and other biological systems has been extensively reviewed in recent years (Magnusson, 1980, 1982; Rutter, 1980; Edebo *et al.,* 1980; Ochoa, 1978). Several alternate approaches to explaining hydrophobic interactions have been taken by various investigators. The present section is thus an attempt to illustrate several viewpoints that have gained some consensus.

One definition for hydrophobic interactions involves the free energy

Table I. Hydrophobicity and Adhesion in Nonbacterial Systems

Phenomenon	Reference
Adhesion of poliovirus to membrane filters	Shields and Farrah (1983)
Foam separation of virus	Morrow (1969)
Adhesion of fungal spores *(Colletotrichum lindemuthianum)* to polystyrene and plant surfaces	Young and Kauss (1984)
Adhesion and growth of yeast on hydrocarbons	Nakahara *et al.* (1981), Miura *et al.* (1977), McLee and Davies (1972), Kappeli and Fiechter (1976)
Sexual interactions of *Paramecium caudatum*	Kitamura (1984)
Invasion of *Plasmodium falciparum* into erythrocytes	Breuer *et al.* (1984)
Adhesion to connective tissue	Wang (1974)
Adhesion properties of tissue culture cells	Malmqvist *et al.* (1984), Reuveny *et al.* (1983)
Adhesion of tumor cells	Hills (1984b)
Adhesion and surface properties of erythrocytes	Todd and Gingell (1980), Neumann *et al.* (1983)
Partitioning of sake yeast at the air–water interface	Ouchi and Akiyama (1971)

change ΔG associated with the process of bringing two entities from infinite separation to distances of the order of magnitude of molecular dimensions (Ben-Naim, 1977). In our case, this free energy change relates to the layering of water molecules at cell and substratum surfaces. According to Ben-Naim (1977), a nonpolar moiety introduced into an aqueous phase will be surrounded by water molecules whose structure is greater than that of molecules in the bulk aqueous phase. Such structured water layer(s) are associated with a decrease in entropy ($\Delta S < 0$) related to the increased order of the system. Thus, in order to solubilize nonpolar solutes, a "hole" must be created in the water structure of the bulk phase; the ensuing breaking of bonds between water molecules demands an input of energy. The formation of new hydrogen bonds in such a process leads to a negative but very small enthalpy change ΔH. Thus, the energy input required to carry out this process may be attributed to the negative entropy change.

What occurs when two hydrophobic surfaces covered by structured water molecules approach one another? In such a case, layers of structured water molecules are released into the bulk aqueous phase from the contacting surfaces. This situation is illustrated schematically in Fig. 2. The freeing of such molecules brings about the reverse situation to that described above: a decrease in free energy due to reduction of the overall nonpolar–water interface by the transfer of water molecules to the less ordered bulk water state (i.e., increase in ΔS). This premise, based on the behavior of water molecules surrounding apolar "cavities" (Arakawa *et al.,* 1979), rests on experimental evidence suggesting that contributions from formation of surface–surface and breaking of surface–water interactions can largely be neglected. The free energy for hydrophobic interactions thus has its largest contribution from the cavity effect, i.e., the entropy function.

Several properties of hydrophobic interactions can serve as criteria for their participation in adhesion of two surfaces. Hydrophobic interactions generally increase with ionic strength due to the suppression of electrostatic interactions (Ochoa, 1978). To a certain point, hydrophobic interactions increase with increasing temperature (Ben-Naim, 1977). Temperature may, however, also provoke increased solvation and decreased surface tension, which counteract the hydrophobic interactions. Hydrophobic interactions are also expected to increase with increasing apolar nature of one or both surfaces under consideration. Hydrophobic interactions are also enhanced by salting-out ions, according to the Hofmeister series (Lindahl *et al.,* 1981). At high concentrations of salting-out ions, the solubility of the solute is adversely affected by decreasing availability of water molecules in the bulk and increasing surface tension of water, resulting in an increase in hydrophobic interactions (Tanford, 1973). The increase in surface tension of water in the presence of salting-out agents may be indicative of increased water structure at low-energy (hydrophobic) interfaces (Tanford, 1973); freeing of increasingly structured water upon the approach of two hydrophobic surfaces, as discussed above, would, in turn, be increasingly favored. The effect of structure-breaking (chaotropic) ions may be viewed as the opposite case; the new ordering of water molecules that they induce prevents them from undergoing further positive entropy changes (Ochoa, 1978).

The above descriptions of hydrophobic interactions are derived from considerations of the close approach of extremely apolar moieties, such as two molecules of alkane. The role of hydrophobic interactions in bacterial adhesion is obviously far more complicated. The term "hydropho-

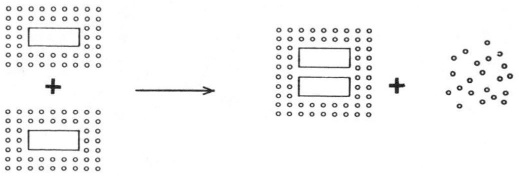

Figure 2. Aggregation of two nonpolar solutes due to hydrophobic interaction. When two separated polar solutes (represented by rectangles, left) covered by ordered layers of water molecules (represented by circles) interact, water molecules are released into the bulk aqueous phase (right). This interaction is accompanied by an increase in ΔS and a smaller increase in ΔH.

bic bacteria" is probably a misnomer: the great majority of bacteria referred to in this chapter are wetted by water and are readily suspended in aqueous medium due to polar, hydrophilic moieties, which abound on the bacterial cell surface. In fact, the ability of certain bacteria to adhere to oil droplets and prevent their coalescence (Rosenberg *et al.,* 1980a) is akin to oil emulsification by amphipathic molecules, which interact with both the water and oil phases. Similarly, the surfaces to which bacteria adhere often contain both polar and nonpolar constituents. The degree of each will influence the extent and structure of the proximal water molecule layers and thus determine the energetic favorability of adhesion, as previously discussed. Thus, it is the interplay between hydrophilic and hydrophobic surface components that determines the overall contribution of hydrophobic interactions to adhesion processes.

The outcome of an adhesion process can be predicted by surface thermodynamics (i.e., the the interfacial tensions between the adhering surfaces), as compared with that of each individual surface with the aqueous medium. Such interfacial tensions are themselves a reflection of the free energy interrelationships between the surfaces in question and the water molecules surrounding them. Development of such a model with respect to bacterial adhesion can be found in Absolom *et al.* (1983). These authors obtained two sets of experimental results; one set consisted of the interfacial tensions of the cells themselves; the second set consisted of adhesion measurements of the cells to surfaces of varying interfacial tensions in the presence of varying concentrations of surfactant (dimethylsulfoxide, DMSO). According to the concept put forward by these investigators, adhesion is independent of substratum surface energy when the surface energies of bacterium and suspending medium are equal. The results obtained for the surface energies of the bacteria tested based on their adhesion correlated well with the surface energies calculated from contact angle measurements. As predicted by their model, adhesion to hydrophilic surfaces was more pronounced only when the bacterial surface tension was larger than that of the surrounding aqueous medium; conversely, adhesion to increasingly hydrophobic surfaces occurred when the surface tension of the aqueous phase was higher than that of the bacterial cells. In many ecosystems, it is the second situation that prevails. In all experiments, moreover, adhesion was highest for the most hydrophobic bacteria for every aqueous surface tension studied.

3. Methods for Determining Cell-Surface Hydrophobicity

The methodology for studying the cell-surface hydrophobicity of bacterial cells can be divided into several groups, according to the type of parameters measured: (1) methods based on adhesion of cells to solid surfaces, at interfaces, or to one another; (2) methods based on the partitioning of hydrophobic molecules between the cells and surrounding medium; (3) methods based on partitioning of cells between two immiscible liquid phases; (4) techniques based upon properties of cell layers; and (5) indirect methods, such as those based on the inhibition of adhesion by agents that interfere with hydrophobic interactions.

Some general factors pertain to most methods currently employed: (1) all materials should be scrupulously clean and free of surfactant contaminants; (2) growth conditions and preparation of bacterial cells should be carefully monitored; and (3) duration of the assay should be minimal, to prevent release of surface components that may have an inhibitory effect, e.g., by "conditioning" of the attachment surface (Fletcher and Marshall, 1982b), or alternatively, synthesis of cell-surface components that alter surface properties.

3.1. Cell-Surface Hydrophobicity Methods Based on Adhesion

In studies related to the role of hydrophobic interactions in bacterial adhesion, methods that are themselves based on adhesion are sometimes the most pertinent. It should be reemphasized that in cases in which adhesion is mediated by a small portion of the bacterial cell, e.g., in partitioning of *Hyphomicrobium, Flexibacter,* and *Rhizobium* cells at the oil–water interface (Marshall and Cruickshank, 1973, Marshall *et al.,* 1975), the surface properties of the large majority of the cell surface make only a small contribution.

3.1.1. Hydrophobic Interaction Chromatography (HIC)

This method, originally developed for protein separation (Hjertén *et al.,* 1974), has found widespread use in many studies of bacterial hydrophobicity. In this technique, aqueous suspensions of Sepharose beads with covalently bound hydrophobic moieties (e.g., phenyl or octyl groups) are usually packed into small columns and the bacterial suspension is subsequently applied. Retention may be determined by various means, such as turbidimetric readings or measurement of colony-forming units (Smyth *et al.,* 1978) or radioactivity (Hermansson *et al.,* 1982). In many cases, salting-out agents are added to promote adhesion to the gel (Smyth *et al.,* 1978). In some cases, adherent cells can be desorbed by lowering the ionic strength of the eluent or by adding detergent (Magnusson *et al.,* 1979a). Since it has been demonstrated that bacteria can become nonspecifically trapped within the gel matrix or between the gel and column (Olsson and Westergren, 1982), the assay is preferably performed by mixing of the beads with the bacterial cells, followed by separation of free from bound cells. Moreover, controls should be performed with the corresponding untreated Sepharose beads themselves, as in some cases bacteria may bind to them as well.

3.1.2. Bacterial Adhesion to Hydrocarbons (BATH)

As already mentioned, bacterial partitioning at the oil–water interface has been studied by various investigators since 1924 (Mudd and Mudd, 1924a,b; Marshall and Cruickshank, 1973; Marshall *et al.,* 1975), including many researchers in the field of petroleum microbiology (e.g., Neufeld *et al.,* 1980; Kennedy *et al.,* 1975; Kirschner-Zilber *et al.,* 1980, Nakahara *et al.,* 1981). In 1980, bacterial adhesion to hydrocarbons (BATH) was suggested as a simple and convenient assay for measuring bacterial hydrophobicity (M. Rosenberg *et al.,* 1980a). In this test, washed bacterial suspensions are vortexed under controlled conditions with an aliphatic (e.g., hexadecane) or aromatic (e. g., xylene) liquid hydrocarbon. In the case of adhesion, bacteria bind to the oil droplets and rise with them following the mixing procedure, bringing about a concomitant drop in the cell density of the lower bulk aqueous phase. This technique and various modifications have been used to study bacterial hydrophobicity in a wide variety of bacterial species and cell mixtures as a function of growth conditions and other parameters (M. Rosenberg, 1984a). The basic technique has also enabled enrichment of hydrophobic mutants in *Acinetobacter calcoaceticus* (M. Rosenberg and Rosenberg, 1981), *Serratia marcescens* (M. Rosenberg, 1984c), and *Streptococcus sanguis* (Gibbons *et al.,* 1983).

One of the main limitations of the method as employed thus far is its semiquantitative nature. A quantitative approach based on the kinetics of adhesion to hexadecane has recently been employed (D. Lichtenstein, M. Rosenberg, N. Sharfman, and I. Ofek, in preparation).

Results obtained using BATH usually correlate with those obtained using other techniques (M. Rosenberg, 1984a). An interesting anomaly in this regard is that enterobacterial strains, which appear to exhibit cell-surface hydrophobicity with other methods, adhere poorly or not at all to hexadecane. However, it was recently shown that adhesion of a rough *Escherichia coli* strain to hexadecane was promoted from 0 to 76% by adding ammonium sulfate to the bacterial suspension (M. Rosenberg, 1984b). This finding suggests that many enterobacterial strains purported to possess pronounced cell-surface hydrophobicity are actually much less hydrophobic than strains that adhere to hydrocarbons in the absence of salting-out agents, e.g., *Streptococcus pyogenes, Acinetobacter calcoaceticus,* and *Serratia marcescens.*

Several points concerning the BATH assay require further clarification: (1) Might the vortexing procedure in the presence of hydrocarbons extract hydrophobic cell-surface components into the hydrocarbon phase, altering the properties measured? (2) Morisaki (1983, 1984) has reported that hydrophobic substrata can affect bacterial metabolism. Do such effects similarly influence adhesion to hydrocarbons? (3) Does cell–cell cooperativity play a role in BATH?

Adhesion to hydrocarbons is discussed further in Section 5.2 in the context of bacterial growth on oil.

3.1.3. Salting-out Aggregation Test (SAT)

This technique, described by Lindahl *et al.* (1981), is based on the premise that the same laws governing precipitation of protein molecules from aqueous solution hold true for aggregation of bacterial cells, i.e., that the more hydrophobic the cell, the greater its tendency to precipitate out of the solution (i.e., through cell–cell interactions) at a lower concentration of salting-out agents. In this method, bacterial cells are suspended in dilute phosphate buffer, and ammonium sulfate is added until aggregation occurs. This technique appears to correlate with other methods in most, but not all, instances (Ferreiros and Criado, 1984). Configurational changes of cell-surface structures due to the high salt concentrations may introduce errors in measurement.

3.1.4. Adhesion to Solid Surfaces

Adhesion to solid surfaces of relatively low surface free energy has been employed to test for bacterial hydrophobicity by a variety of investigators. The ability of cells from bacterial colonies to adhere directly to polystyrene has been employed as a qualititative technique for screening hydrophobic colonies (M. Rosenberg, 1981), and, more recently, as a rapid agglutination test (Lachica and Zink, 1984b). Various investigators have used adhesion to polystyrene or similar surfaces as a quantitative measure of bacterial hydrophobicity, measuring the number of adherent cells by a variety of techniques (e.g., direct counting, measurement of absorbed stain). Adhesion to solid surfaces of varying surface energies has also been studied (Gerson and Akit, 1980; Fletcher and Loeb, 1979; Dexter *et al.,* 1975; Dexter, 1979; Pringle and Fletcher, 1983). The latter authors observed that 16 freshly collected bacterial isolates adhering to plastic surfaces in freshwater exhibited the highest attachment to more hydrophobic surfaces. Absolom *et al.* (1983) have used an elegant technique (originally developed for measuring hydrophobic properties of mammalian cell surfaces) for the measurement of bacterial hydrophobicity (see Section 2). Bacteria suspended in different concentrations of

DMSO are brought into contact with smooth, solid surfaces of varying surface free energies. Using this method, these workers have been able to ascribe surface free energies to several bacteria tested. Unfortunately,the bacterial suspensions all fell within a narrow range of surface energy values; thus, it is difficult to predict whether this technique would be similarly successful with strains generally considered to be substantially more hydrophobic.

3.2. Molecular Probes

Dyar (1948), and later Hill *et al.* (1963), employed charged surfactant molecules in order to determine cell-surface hydrophobicity in staphylococcal and streptococcal cells. In such studies, small concentrations of ionic surfactant (usually negatively charged, to eliminate electrostatic interactions between positively charged probes and the net negative charge of most bacterial surfaces) were added to bacterial suspensions. Changes in the electrophoretic mobility of the cells were attributed to binding of surfactant molecules via hydrophobic interactions to the cell surface.

This principle was employed by Kjelleberg *et al.* (1980), who measured binding of radiolabeled dodecanoic acid to bacterial cells. Analysis of the results from several strains indicated that bacteria may differ in the number of binding sites available for attachment of hydrophobic molecules as well as in the affinity of such bonds. This method can yield important information concerning the nature and number of hydrophobic binding sites on the cell surface. A similar approach using palmitic acid has been proposed by Malmqvist (1983). The main concern in the use of such hydrophobic probes is their small size, with the possibility that they can intercalate within the cell envelope, thus binding to sites that are not normally exposed at the outermost cell surface.

3.3. Contact Angle Measurements (CAM)

Contact angle measurements are routinely employed by surface scientists to measure surface properties of clean, smooth, flat, dry, solid surfaces. Small drops of liquid are applied to the surface under controlled conditions and the angle of contact recorded directly. Recently, van Oss and coworkers have proposed that such measurements, conducted on bacterial layers, can provide quantitative information on the hydrophobicity of the individual cells (van Oss and Gillman, 1972; Cunningham *et al.,* 1975; van Oss, 1978), including a value for the surface energy of the bacteria measured (Absolom *et al.,* 1983). Potential drawbacks of the methodology include: (1) the cells must remain moist, but not wet; thus, results must be obtained within a given time during the drying out process of the layer; (2) the surface, being composed of individual cells, is not flat; and (3) it is not known whether the surface presented by the outermost bacterial layer (facing upward) provides an overall picture or is the result of an alignment of cells, perhaps each projecting a patch of increased or decreased hydrophobicity. Despite these reservations, the results obtained using this method have been corroborated by other, independent techniques (Absolom *et al.,* 1983). Fletcher and Marshall (1982a) have introduced a modification (albeit, for studying substrata) in which the contact angle of air bubbles surrounded by water on the inverted cell surface is measured. This method was employed by Fattom and Shilo (1984) to study the hydrophobicity of Cyanobacteria. Similarly, Neufeld *et al.* (1980) have suggested replacing the air surrounding the water droplet by oil. Whereas most studies have employed drops of water or saline, Busscher *et al.* (1984) calculated the surface energies of oral bacteria based on contact angles of water, water–in-propanol mixtures, and α-bromonapthalene.

3.4. Two-Phase Partitioning (TPP)

Several decades ago, Albertsson (1958) proposed a method for particle separation based on partitioning between two polymeric aqueous phases that are mutually immiscible, such as dextran and polyethylene glycol. Later, this technique was used to separate bacterial cells; e.g., smooth and rough *Salmonella typhimurium* cells [reviewed in Edebo *et al.* (1980)]. Hydrophobicity of the polyethylene glycol phase can be enhanced by the covalent binding of acyl groups (Magnusson and Johansson, 1977), enhancing the affinity of hydrophobic bacteria for this phase as compared to the dextran layer. One possible problem associated with this method is that a portion of the bacteria may become trapped between the two phases (Sacks and Alderton, 1961), complicating recovery and interpretation. Nonetheless, this technique may be viewed as being relatively nondestructive, and avoids the use of high concentrations of salts or organic solvents. Gerson and Akit (1980) reported linear correlations between the TPP and CAM methods, enabling them to grade the relative hydrophobicity of several bacterial species.

3.5. Indirect Methods

Hydrophobic interactions are inhibited by a wide range of molecules, including those that exhibit surface-active and/or chaotropic activities. In some cases, a role for hydrophobic interactions in the mediation of adhesion phenomena may be invoked based on the ability of such compounds to inhibit or reverse adhesion (Erne *et al.,* 1984; Nesbitt *et al.,* 1982). For example, adhesion of *Thiobacillus* to sulfur granules is inhibited by sodium dodecyl sulfate (Bryant *et al.,* 1984). Adhesion of *Pseudomonas aeruginosa* to erythrocytes is inhibited by chaotropic agents, but not by various sugars, suggesting the involvement of hydrophobic interactions (R. J. Doyle, personal communication). Inhibition of bacterial autoaggregation and coaggregation by detergents has underlined the importance of hydrophobicity in these phenomena (McIntire *et al.,* 1982; Stanley and Rose, 1967). Surface-active alcohols are often potent adhesion inhibitors (M. Rosenberg *et al.,* 1980a; Fletcher, 1983). E. Rosenberg *et al.* (1983a) have proposed that the majority of attached normal microbiota on the surface of human buccal epithelial cells adhere via hydrophobic interactions, since they are desorbed by low concentrations of emulsan, an amphipathic polymer.

4. Surface Components that Influence Bacterial Hydrophobicity

In preparing this section, we encountered the problem of how to denote surface components that promote bacterial hydrophobicity. To call them "adhesins" would presuppose a role in adhesion. In at least some cases, this is apparently not the case (Davison and Sanford, 1982; Kozel, 1983; Sobel and Obedeanu, 1984). We have thus elected to use the term *hydrophobins* to denote cell-surface components that contribute to cell-surface hydrophobicity; similarly, surface components that reduce cell-surface hydrophobicity have been termed *hydrophilins.*

Investigations into the hydrophobic surface properties of *Acinetobacter calcoaceticus* RAG-1 illustrate the possible complexity involved in determining the participating cell-surface components. Several lines of evidence establish a role for thin fimbriae as hydrophobins in RAG-1: (1) MR-481, a nonhydrophobic mutant of RAG-1, is deficient in thin fimbriae, (2) partial adhesion revertants possess numerous thin fimbriae, (3) shearing of fimbriae reduces hydrophobicity in RAG-1 cells grown on minimal medium, and (4) strain AB-15, an independent, thin fimbria-less mutant, is nonadherent when grown on minimal medium (M. Rosenberg *et al.,* 1982a). Thin fimbriae, however, are not the whole story, since (1) AB-15 cells are hydrophobic following growth on complex medium (despite their total lack of thin fimbriae); and (2) crossed immunoelectrophoresis of supernatants following sonic oscillation of complex-medium-grown cells reveals that the nonhydrophobic mutant MR-481 lacks at least three surface antigens, in addition to the thin fimbriae, when compared with wild-type RAG-1 cells (E. A. Bayer and M. Rosenberg, unpublished data). These results suggest that thin fimbriae are the main hydrophobin of RAG-1 cells grown on minimal medium, but that additional factor(s) contribute to the hydrophobicity of complex-medium-growth cells. To complicate matters further, the emulsan minicapsule of RAG-1 acts as a hydrophilin (E. Rosenberg *et al.,* 1983b). Complete loss of cell-surface emulsan leads to exposure of hydrophobins, which are apparently masked in wild-type cells (Pines and Gutnick, 1984).

In the light of such investigations, the presumption that cell-surface hydrophobicity is due to a single factor may often be premature. In some instances [e.g., *Aeromonas salmonicida* (Trust *et al.,* 1983, N. D. Parker and Munn, 1984)], bacterial hydrophobicity may indeed be due to a single hydrophobin, which dominates the outer cell surface. This, however, may be the exception rather than the rule. Bacterial hydrophobicity may often involve a delicate interplay of charged and uncharged, polar and apolar groups, exposed to varying degrees, and with varying densities over the cell surface (Kjelleberg, 1984b). Moreover, it is impossible to determine precisely where the cell surface ends and the surrounding aqueous phase begins. On a molecular level there is no sharp boundary at the outermost cell surface, and water molecules can penetrate the outer bacterial layers to varying extents. In some cases, surface projections, such as various fimbriae, fibrils, holdfasts, and so on, may themselves mediate adhesion to hydrophobic surfaces (Marshall and Cruickshank, 1973), in which case the cell itself may be tethered to the surface.

Components of the bacterial cell surface usually vary greatly as a function of growth conditions, including parameters such as aeration, temperature of growth, growth medium, and age of cells. Thus, whereas some investigators originally proposed that bacterial strains could be assigned cell-surface hydrophobicity values independent of growth conditions (van Oss and Gillman, 1972), more recent studies have shown that hydrophobic surface properties vary greatly as a function of physiological conditions (M. Rosenberg, *et al.,* 1980a, 1981; Kjelleberg *et al.,* 1980; Ofek *et al.,* 1983; Nesbitt *et al.,* 1982; Rogers *et al.,* 1984). The dramatic increase in hydrophobicity of *A. calcoaceticus* RAG-1 cells over the course of several generations of growth is illustrated in Fig. 3. Starvation effects also have a large influence on cell-surface hydrophobicity (Kjelleberg, 1984a; Kjelleberg *et al.,* 1983; Kjelleberg and Hermansson, 1984). The large differences in hydrophobicity of starved versus fed natural populations are shown in Table II. Finally, it must be emphasized that cell-surface hydrophobicity varies not only from species to species, but also from strain to strain (M. Rosenberg, 1982; Olsson and Westergren, 1982). Thus, within a given species, various combinations of hydrophobins may be responsible for the hydrophobic properties observed. Indeed, this may account for arguments such as whether the hydrophobicity of group A streptococci is mediated by lipoteichoic acid (Miorner *et al.,* 1983, 1984),

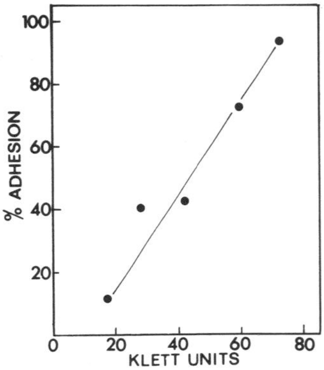

Figure 3. Adhesion to hexadecane as a function of growth. *Acinetobacter calcoaceticus* RAG-1 cells were grown in modified nutrient broth with shaking at 30°C. At various growth culture turbidities samples were removed. Cells were washed and assayed for adhesion to 0.2 ml of hexadecane as described (M. Rosenberg *et al.*, 1980a). Results are expressed as percent adhesion to hexadecane as a function of growth culture turbidity.

M protein (Tylewska *et al.*, 1979), or both (Courtney *et al.*, 1983; Ofek *et al.*, 1983; Wadstrom *et al.*, 1984); all these possibilities, and more, may be found in various strains.

The dependence of hydrophobic properties on physiological conditions can often be correlated to the synthesis, or degree of exposure, of hydrophobins and hydrophilins, as in the case of *Streptococcus pyogenes* (Ofek *et al.*, 1983). The presence of plasmids may similarly affect cell-surface hydrophobicity by allowing expression of fimbriae and other surface components (Lindahl *et al.*, 1981; Lachica and Zink, 1984a; Martinez, 1983; Ferreiros and Criado, 1984). Finally, mechanical, enzymatic, or other destructive treatments of the cell surface can alter hydrophobicity. These include studies in which buried hydrophobins are unmasked (e.g., Mackey, 1983), resulting in increased hydrophobicity, as well as those in which hydrophobins are removed, e.g., by proteolytic enzymes (Ofek *et al.*, 1983) or mechanical means (M. Rosenberg *et al.*, 1982a). The development of cell-surface hydrophobicity in *Escherichia coli* and *Sal-*

Table II. Adhesion to Octyl–Sepharose Beads and Hexadecane of the Total Microbiota of the Colon Content of Fed and Starved Flounder[a]

	Percent adhesion[b]	
Animals	To octyl–sepharose	To hexadecane
Fed	3	16
Starved	74	53

[a] P. L. Conway, J. Maki, R. Mitchell, and S. Kjelleberg (unpublished results).

[b] Percentage adhesion was measured following mixing and equilibration. Results are expressed as the percentage drop in the number of the cells remaining in the aqueous phase following the respective assays.

monella typhimurium following invasion by *Bdellovibrio* cells is an example of bacteria-inflicted damage resulting in changes in surface hydrophobicity (Cover and Rittenberg, 1984).

Several cell-surface components identified as hydrophobins in various bacteria are listed in Table III. The following sections deal with some of these examples in greater detail.

4.1. Fimbriae and Surface Fibrils

Fimbriae (or surface fibrils) have often been cited as hydrophobins. In some cases [e.g., the thick fimbriae of *Acinetobacter calcoaceticus* RAG-1 (M. Rosenberg *et al.,* 1982a)] fimbriae play no role in modulating cell-surface hydrophobicity and may even act as hydrophilins, as in the

Table III. Some Proposed Bacterial Hydrophobins

Component	Bacteria	Reference
Thin fimbriae	*Acinetobacter calcoaceticus* RAG-1	M. Rosenberg *et al.* (1982a)
M protein, lipoteichoic acid, or both	Group A streptococci	Ofek *et al.* (1983), Miorner *et al.* (1983), Wadstrom *et al.,* (1984), Tylewska *et al.* (1979)
A protein	*Staphylococcus aureus*	Jonsson and Wadstrom (1983, 1984)
"A layer" protein	*Aeromonas salmonicida*	Trust *et al.* (1983), N. D. Parker and Munn (1984)
Prodigiosin	Pigmented *Serratia* strains	Blanchard and Syzdek (1982), Hermansson *et al.* (1979), Kjelleberg *et al.* (1980)
Glucosyltransferase	*Streptococcus mutans*	D. Oakley and R. J. Doyle (unpublished results)
Outer membrane proteins	*Vibrio cholerae*	Kabir and Ali (1983)
Surface fibrils	*Streptococcus sanguis*	Gibbons *et al.* (1983)
	Yersinia enterocolitica	Lachica and Zink, (1984 a,b), Lachica *et al.* (1984)
Various fimbriae	*Escherichia coli*	Faris *et al.* (1981), Smyth *et al.* (1978), Honda *et al.* (1984), Jann *et al.* (1981), Svanborg-Eden *et al.* (1984), Ohman *et al.* (1982)
Core oligosaccharides or outer membrane lipids	Rough enterobacterial strains	Edebo *et al.* (1980), M. Rosenberg *et al.* (1982b)
Gramicidin S	*Bacillus brevis* spores	E. Rosenberg, *et al.,* 1985.

case of *Serratia* (Hermansson *et al.,* 1982). All, or almost all, of the fimbriae examined to date contain a high proportion of hydrophobic amino acid residues (Jones and Isaacson, 1983). This, however, does not necessarily imply that all fimbriae are hydrophobins, since their ability to undergo hydrophobic interactions would depend on the spatial disposition of these residues (i.e., exposure toward the bulk aqueous phase) rather than their total number. Certainly, among the fimbriae reported to act as hydrophobins, great variations are observed in the cell-surface hydrophobicity that they confer. This, in turn, may also be a function of the number of fimbriae per cell and their topographic distribution.

Of particular interest in this regard are the hydrophobic interactions attributed to mannose-sensitive ("type 1") and various mannose-resistant fimbriae of enterobacterial strains. Although fimbriated *Escherichia coli* strains are usually more hydrophobic than nonfimbriate counterparts, the degree of hydrophobicity that they confer is low. For example, the adhesion of fimbriae-bearing enterobacterial strains in HIC is largely dependent on the presence of high concentrations of salting-out agent (Jann *et al.,* 1981; Ohman *et al.,* 1982; Faris *et al.,* 1981). Moreover, whereas certain fimbriae can promote adhesion to hydrocarbons and polystyrene (M. Rosenberg *et al.,* 1982a), various fimbriated *Escherichia coli* strains do not adhere to either surface (T. Zusman and E. Ron, unpublished results; M. Rosenberg, 1982). Such observations may, however, understate the role of hydrophobic interactions (however weak) on attachment of enterobacteria to host cells, as described in Section 5.7.

The presence of fimbriae is related to bacterial adhesion in natural systems (Pertsovskaya *et al.,* 1973; Romanenko, 1979). Furthermore, it was recently found that hydrophobic fimbriae-bearing *Salmonella typhimurium* cells adhered in higher numbers to several mineral particles than nonfimbriated, less hydrophobic cells (Stenstrom and Kjelleberg, 1985).

4.2. Role of Capsule

In almost all studies [with the exception of some encapsulated staphylococci, according to Hogt *et al.* (1983a)] the presence of bacterial capsule or slime has been shown to decrease surface hydrophobicity. For example, enzymatic removal of the capsule of *Acinetobacter calcoaceticus* BD4 led to an increase in adhesion to hexadecane from 0 to 100% (E. Rosenberg *et al.,* 1983b). Cell-surface capsule reduces cell-surface hydrophobicity in a *Phormidum* sp. (Fattom and Shilo, 1984), and was reported to lower phagocytosis of *Staphylococcus aureus* by rendering the cells more hydrophilic (van Oss and Gillman, 1972). Adhesion of *Streptococcus pyogenes* to hexadecane corresponds to the absence of hyaluronic acid-containing capsule (Ofek *et al.,* 1983). A mutant of a freshwater *Pseudomonas fluorescens* strain producing an exopolysaccharide dominated the aqueous phase, whereas the non-exopolysaccharide-producing wild type was foam-fractionated by the adsorption bubble separation process in a fermentor (Pringle *et al.,* 1983). Recently, Runnels and Moon (1984), employing several strains of encapsulated, fimbriated *Escherichia coli* and their noncapsular mutants, showed that the presence of capsule impeded K99-mediated adhesion.

Rough mutants of smooth enterobacterial strains, such as *Salmonella typhimurium* (Edebo *et al.,* 1980), *E. coli* (M. Rosenberg *et al.,* 1980a; M. Rosenberg, 1984b), and *Proteus mirabilis* (M. Rosenberg *et al.,* 1982b), are more hydrophobic, presumably due to loss of surface oligosaccharides.

Such results suggest the antiadhesive role of capsule, and do not appear to concur with various studies that claim a major role for capsule in mediating adhesion to a wide variety of substrata (Costerton *et al.,* 1981). However, since the latter experiments are usually conducted over the course of several hours or days, the copious amounts of polysaccharide observed at attachment sites may or may not be involved in the initial attachment process. We therefore suggest that whereas capsules may act to bridge and cement layers of adhering bacteria to substrata, they are not necessarily involved in initial adhesion processes. This, however, does not apply for polysaccharide layers undergoing gel formation (Rutter, 1980).

4.3. Prodigiosin and Serratial Hydrophobicity

Prodigiosin is the cell-surface pigment that accounts for the red coloration of *Serratia* strains. Various investigators have found correlations between the appearance of prodigiosin and cell-surface hydrophobicity (M. Rosenberg *et al.,* 1980a; Kjelleberg *et al.,* 1980; Blanchard and Syzdek, 1978; Hermansson *et al.,* 1979; Syzdek, 1985), implicating this molecule as a hydrophobin in pigmented strains. However, several studies have shown that nonpigmented strains and mutants may also be substantially hydrophobic (Hermansson *et al.,* 1979; M. Rosenberg, 1984c; M. Rosenberg and Y. Blumberger, unpublished data). Moreover, a pigmented, nonhydrophobic *S. marcescens* mutant was recently isolated (M. Rosenberg, 1984c). This mutant differs from wild-type cells in the increased translucence of its colonies, suggesting that factor(s) conferring colony opacity are additional (and perhaps crucial) hydrophobins in pigmented cells, and may similarly contribute to cell-surface hydrophobicity in nonpigmented strains. In contrast to reports linking mannose-sensitive adhesins with cell-surface hydrophobicity in *E. coli* (Jann *et al.,* 1981; Ohman *et al.,* 1982), no relationship was found between mannose-sensitive aggregation of yeast cells and hydrophobicity in clinical *Serratia* isolates (M. Rosenberg, and Y. Blumberger, unpublished results).

4.4. Cell-Surface Hydrophobicity in Bacterial Spores

Increased hydrophobicity of *Bacillus* spores as compared to that of vegetative cells has been shown by increased adhesion to hydrocarbon (M. Rosenberg, 1984a; Doyle *et al.,* 1984) and partitioning in an aqueous two-phase system (Sacks and Alderton, 1961) and at the air–water interface (Boyles and Lincoln, 1958; Gaudin *et. al.,* 1960). Cell-surface hydrophobicity in spores may provide a biological advantage in enabling their dispersal via aerosols (see also Section 5.3). However, increased hydrophobicity of spore versus vegetative cells is not universal: Kupfer and Zusman (1984) have recently reported that sporulating *Myxococcus xanthus* cells become less and less hydrophobic as sporulation proceeds.

A possible role for the antibiotic gramicidin S in mediating the hydrophobicity of *B. brevis* spores has been recently demonstrated (E. Rosenberg *et al.*). Whereas wild-type spores, which produce the antibiotic, were hydrophobic, gramicidin S-negative mutant spores were not; however, extraneous addition of gramicidin S increased the hydrophobicity of the mutant spores in a dose-dependent manner. These data suggest that the cationic moiety of gramicidin S binds to anionic receptors on the spore surface, exposing the hydrophobic amino acid residues of the antibiotic molecule on the cell surface.

5. Hydrophobicity and Adhesion in the Environment and Host

5.1. Introduction

In the present section we deal with the role of hydrophobic interactions in bacterial adhesion to a variety of surfaces in the open environment as well as in the host. Whereas most *in vitro* investigations deal with adhesion of washed bacterial suspensions to clean surfaces, the *in situ* situation is usually quite different. Interfaces in aqueous surroundings are rapidly coated with a "conditioning" layer of adsorbed macromolecules and lipids (Fletcher, 1976); Fletcher and Marshall (1982a) proposed a useful method for *in vitro* measurement of the interfacial properties of such films, which may profoundly affect bacterial adhesion (Kjelleberg and Stenstrom, 1980). Morever, bacteria (particularly within animal hosts) adsorb, whether specifically or not (Miorner *et al.,* 1980; Absolom *et al.,* 1982), antibodies and other macromolecules (Wadstrom *et al.,* 1984). This, in turn, either promotes or inhibits the ability of the cell to attach, depending on the cell-surface hydrophobicity of the cell prior to adsorption and the nature of the adsorbing molecules (van Oss, 1978). The *in situ* coating of the bacterial surface and the attachment substratum is generally difficult to estimate, making it hard to extrapolate from laboratory experiments to *in situ* adhesion. Several strategies in this direction would include the following: (1) Comparision of the ability of laboratory strains to adhere to the actual surface under study as a function of their relative hydrophobic surface properties. One such study by Svanberg *et al.* (1984) demonstrated that hydrophobic *Streptococcus mutans* is better able to attach to and colonize oral surfaces of human volunteers as compared to a nonhydrophobic variant; (2) attempting to desorb attached bacteria by means of agents that interfere with the mechanism under study; and (3) direct examination of the adherent properties of bacteria isolated directly from the niche under study. This last point is of great importance in the light of experiments showing that cell-surface hydrophobicity may be dependent on the degree of starvation of cells (Kjelleberg and Hermansson, 1984), strain history (Westergren and Olsson, 1983), and other factors that differ widely between cells in the environment and in the test tube. With few exceptions (e.g., M. Rosenberg *et al.*, 1983c), the cell-surface hydrophobicity of bacteria taken directly from nature and tested without further growth has not been investigated. This is unfortunate, since such experiments are not difficult to perform (some results on hydrophobicity of bacteria taken directly from gastrointestinal systems appear in Table II and in Section 5.6).

5.1.1. "Specific" versus "Nonspecific" Hydrophobicity

Various investigations attest to the nonspecific nature of bacterial adhesion to low-energy surfaces. For example, *A. calcoaceticus* RAG-1 adheres to liquid and solid hydrocarbons, plastic surfaces, human teeth, buccal epithelial cells and air bubbles, exposed metal surfaces, and so on (M. Rosenberg, 1982, and unpublished observations). Thus, it is not surprising that many researchers agree that hydrophobic interactions that mediate bacterial adhesion are not dependent on molecular complementarity between bacterial surface components and receptors on the attachment substratum or on the "molecular texture" of the two surfaces. However, the possibility has been raised that in adhesion to host tissue, specific recognition mechanisms between hydrophobic moieties and stereospecific receptors do occur (Ofek *et al.*, 1983; Simpson *et al.*, 1980), much in the same way that serum albumin binds hydrophobic molecules. Thus, in the case of adhesion of *S. pyogenes* to buccal epithelial cells, specific bacterial hydrophobins (e.g., M protein–lipoteichoic acid complex) would be recognized by epithelial cell receptor(s) (e.g., fibronectin) and adhesion mediated by this specific mechanism (Abraham *et al.,* 1983; Courtney *et al.,* 1983). It is also possible that hydrophobic interactions, in addition to their ability to mediate adhesion *per se,* may also contribute to stereospecific adhesion mechanisms. In the case of mannose-sensitive adhesion, for example, great importance may be attributed to apolar moieties in the immediate proximity of the mannose-binding site. Firon *et al.* (1982) have reported that mannosides of an increasingly hydrophobic nature possess much higher affinity for the binding site, similar to results obtained with the lectin concanavalin A (Lis and Sharon, 1977). Similarly, the presence of mannose has been reported to decrease surface hydrophobicity of *E. coli* bearing type 1 fimbriae, presumably due to some conformational change at the sugar-binding site (Ohman, 1983). Such data suggest that neighboring hydrophobic interactions may promote mannose-sensitive binding in *E. coli* strains.

5.2. Adhesion and Growth at the Solid–Liquid Interface

The possible role of hydrophobicity and adhesion in the life cycle and survival of bacteria in the environment is discussed in the present section.

Adhesion as a tactic in starvation survival was proposed by Dawson *et al.* (1981). More recently, the existence of an increased degree of irreversible binding and hydrophobicity upon starvation has been demonstrated for some bacterial species (Kjelleberg and Hermansson, 1984); in other bacteria tested, this trend was not observed (Kjelleberg *et al.,* 1985a). Other researchers (Ellwood *et al.,* 1982), studying a diverse microbial community, showed a correlation between carbon limitation and attachment. In this context, it should be emphasized that each organism has its own specific starvation-survival pattern (R. Y. Morita and R. Jones, personal communication).

The role of interfaces as areas of nutrient supply in nutrient-deprived ecosystems has been highlighted (Marshall, 1979). The question of whether bacterial activity at such surfaces is affected by the surface *per se,* by nutrients at the interface, or both, is currently under consideration by various investigators (e.g., Morisaki, 1983, 1984; Jeffrey and Paul, 1985; Fletcher, 1985; Gordon and Millero, 1985). It is noteworthy, however, that the scavenging of surface-localized long-chain fatty acids was favored by a hydrophobic *S. marcescens* strain as compared with its hydrophilic mutant (Kefford *et al.,* 1982). These authors also showed that reversibly bound bacteria, attaching and rapidly desorbing, scavenge surface-localized material. This utilization of surface-immobilized substrates has more recently been shown to be a common feature for a wide range of natural marine isolates (Hermansson and Marshall, 1985); whereas irreversibly bound cells exhibited more efficient utilization, reversibly bound bacteria, which are prevalent in the environment, were also able to utilize surface-immobilized materials.

Based on several investigations (e.g., Dawson *et al.,* 1981; Kjelleberg *et al.,* 1982; Kjelleberg and Hermansson, 1984; Hermansson and Dahlback, 1983; Pedros-Alio and Brock, 1983), Dahlback (1983) suggested a life cycle in which planktonic, starved hydrophobic bacteria adhere readily to available interfaces, efficiently scavenge surface-localized materials, increase in biomass and size, and then leave the interface, usually in the form of a daughter cell following division (Kjelleberg *et al.,* 1982) to face the nutrient-deprived conditions in the planktonic phase. The mechanism for bacterial desorption from the interface may involve changes in cell-surface hydrophobicity or, alternatively, localized bacterial production of amphipathic materials that remove bound cells (E. Rosenberg *et al.,* 1983a).

5.3. Adhesion and Growth at the Oil–Water Interface

Adhesion of bacteria and yeast to hydrocarbons has often been observed by microbiologists interested in the field of oil degradation, but such investigators considered this property to be specific to oil-degrading microorganisms (Kennedy *et al.,* 1975; Miura *et al.,* 1977), ignoring several previous investigations (Mudd and Mudd, 1924a; Marshall and Cruickshank, 1973). M. Rosenberg *et al.* (1980a,b) showed that oil-degrading bacteria may or may not adhere to hydrocarbons, and that nondegraders (e.g. *Serratia marcescens, Staphylococcus aureus*) may adhere in high proportions despite their inability to metabolize hydrocarbon. These results suggested the use of bacterial adhesion to liquid hydrocarbons as a general technique for determining cell-surface hydrophobicity (Section 3.1.2) (M. Rosenberg *et al.,* 1980a,b.) Another interesting result of this study was that adhesion of *Acinetobacter calcoaceticus* RAG-1 is not limited to hydrocarbons that it metabolizes. Later studies (M. Rosenberg and Rosenberg, 1981; M. Rosenberg *et al.,* 1982a) addressed the possible role of adhesion in enabling growth of RAG-1 on hexadecane. RAG-1 cells grew readily on hexadecane under conditions of limited hydrocarbon emulsification (low initial cell density and mild agitation), whereas its nonadherent mutant, MR-481, did not grow unless emulsifier was added. Moreover, prolonged incubation of MR-481 cells in hexadecane growth medium led to the eventual growth of increasingly adherent revertants (M. Rosenberg *et al.,* 1982a; Pines and Gutnick, 1984). In a comparison of the adhesion of RAG-1 with that of an oil-degrading strain of *Pseudomonas aeruginosa,* the latter strain exhibited extremely poor affinity for hydrocarbons, even following growth on hexadecane (M. Rosenberg *et al.,* 1980b; Nakahara *et al.,* 1981). These results suggest that nonadherent strains of bacteria that grow on hydrocarbons in the laboratory (i.e., in closed systems that enable emulsification of the insoluble substrate) will not grow well on oil *in situ.* In the open environment, adhesion to oil is frequently observed (e.g., Wyndham and Costerton, 1982); however, emulsification of oil droplets in the open waters would allow them to diffuse away from the cells. Some investigators (working primarily with *Pseudomonas* strains) have argued that adhesion is not an important factor in growth on oil (Reddy *et al.,* 1982; Chakravarty *et al.,* 1975). This may well be true in the case of growth on aromatic hydrocarbons, which are generally more water-soluble and more toxic than paraffins.

An interesting aspect of growth on hydrocarbons is the relationship between bound and free cells. Kirschner-Zilber *et al.* (1980) studied adhesion and growth of a marine pseudomonad on solid *n*-tetracosane; the investigators proposed that individual cells could alternate between the water–paraffin interface and the bulk aqueous phase, with a continual flux of adhering and desorbing cells. The appearance of free *A. calcoaceticus* RAG-1 cells during growth on hexadecane is not due to their inability to adhere to hexadecane, but probably results from their desorption by emulsan, the amphipathic polysaccharide released by the cells (M. Rosenberg and Rosenberg, 1981; E. Rosenberg *et al.,* 1983a). It has been shown (M. Rosenberg, 1982) that bacteria adhering to hexadecane can be desorbed following freezing and thawing of the hydrocarbon phase (Fig. 4). This phenomenon may reflect a basic difference between adhesion at deformable (liquid–liquid and liquid–gas) and relatively nondeformable (liquid–solid) surfaces (P. Rutter, personal communication). Assuming that bacterial adhesion at the oil–water phase does entail gross deformation of the interface [as proposed by Mudd and Mudd (1924b)], the phase transition from liquid to solid hexadecane may reduce the surface available for interaction and thus decrease the energetic favorability of adhesion, with the resultant desorption of cells. The observation that oils readily remove bound bacteria from polystyrene (M. Rosenberg *et al.,* 1983b) may be related to preferential partitioning of bacteria at liquid–liquid rather than liquid–solid interfaces.

Another interesting observation is that bacteria often tend to adhere to oil droplets in patches, both during growth on oil (E. Rosenberg and M. Rosenberg, unpublished data) and following the adhesion assay. Such patches may arise from cell–cell interactions at the interface. Further evidence for this possibility is that bacteria desorbed from hexadecane following freezing and thawing of the hydrocarbon phase are recovered in the bulk aqueous phase as aggregates, rather than individual cells (M. Rosenberg, 1982).

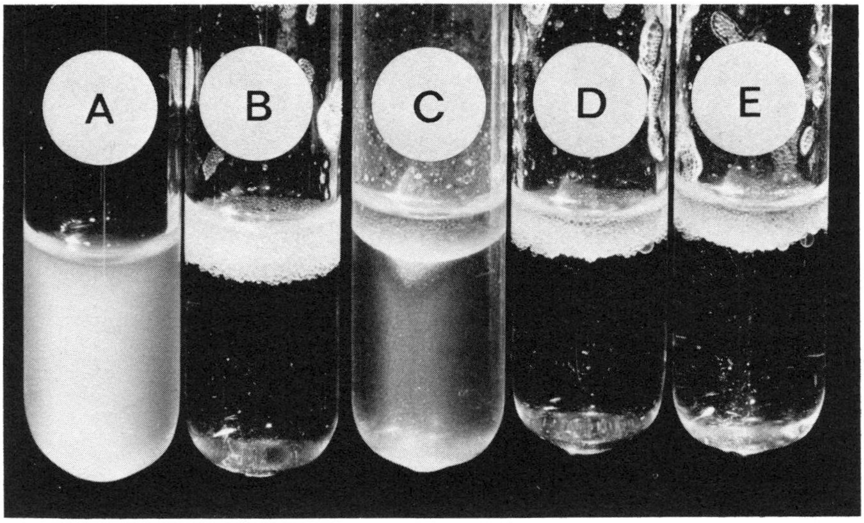

Figure 4. Release of cells bound to hexadecane due to phase transition of hydrocarbon. RAG-1 cells were tested for adhesion to hexadecane (tubes B and C) and octane (tubes D and E) as previously described (M. Rosenberg *et al.*, 1980a). Following the adhesion assay, test tubes C and E were cooled to 12°C for 3 min, then allowed to equilibrate with room temperature (22°C). In the tube containing RAG-1 cells bound to hexadecane (tube C), the hexadecane droplets solidified, and following equilibration at room temperature, melted and coalesced, with concomitant release of clumped RAG-1 cells back into the aqueous phase. No such effect was observed in a similarly treated tube (tube E) containing cells bound to octane (which did not undergo phase transition). Test tube A contains the washed bacterial suspension prior to the adhesion assay. [From M. Rosenberg (1982).]

5.4. Adhesion at Gas–Liquid Interfaces

Mechanisms for adhesion at gas–liquid interfaces have been recently reviewed (Kjelleberg, 1984b, 1985). It appears that cell-surface hydrophobicity is the prime (Hermansson *et al.,* 1982), although not the sole (Kjelleberg and Stenstrom, 1980), criterion in determining attachment at the air–water interface under conditions simulating those present in the environment. A large number of bacteria adhering to the gas–liquid interface of marine waters were tested and found to be significantly more hydrophobic than free-living bulk water bacteria (Dahlback *et al.,* 1980, 1981). Gas–liquid interfaces are not confined in nature to the surface microlayers of oceans and lakes (Norkrans, 1980), but include bubbles rising through the water column and aerosol production by bursting bubbles (Blanchard and Syzdek, 1978, 1982; Bezdek and Carlucci, 1974; B. C. Parker *et al.,* 1983; Wendt *et al.,* 1980; Weber *et al.,* 1983). The significance of such aerosols as microbial carriers has long been recognized (e.g., Woodcock, 1948). Not all bacteria are aerosolized with equal efficiency (Hejkal *et al.,* 1980); mediating factors include the sizes of both cell and bubble, and the cell-surface hydrophobicity (Blanchard, 1983). In the laboratory, adhesion at the air–water interface has proven an efficient means of separating bacteria (Boyles and Lincoln, 1958; Gaudin *et al.,* 1960; Rubin *et al.,* 1966), viruses (Morrow, 1969), and yeast (Ouchi and Akiyama, 1971; Iimura *et al.,* 1980) based on relative hydrophobicity. The latter investigators showed correlations between hydrophobicity and surface pellicle formations. Neufeld and Zajic (1984) have recently demonstrated that partitioning of washed suspensions of *A. calcoaceticus* cells at the air–water interface is accompanied by a significant decrease in surface tension. This finding may lead to an additional means for measuring bacterial hydrophobicity.

5.5. Life Style of Cyanobacteria

Fattom and Shilo (1984) have demonstrated a striking correlation between the mode of growth of aquatic cyanobacteria and their ability to adhere to hydrocarbon. Whereas benthic strains, which grow attached to submerged solid surfaces (sediments, stones, plant surfaces), adhered to hexadecane, free-floating (planktonic) types were invariably nonadherent. The hydrophobic surface properties of benthic cyanobacteria were further demonstrated using other techniques for studying cell-surface hydrophobicity: bubble contact angles on cell layers and adhesion to phenyl–Sepharose beads and to polystyrene. Adhesion of benthic cyanobacteria to hexadecane was dependent on the presence of divalent cations in the assay buffer. Fattom and Shilo also isolated mutants of the hydrophilic planktonic strain *Spirulina platensis* that exhibited increased hydrophobicity. Of particular interest are induced changes in hydrophobicity in *Phormidium* that can be correlated ultrastructually to changes in the cell surface (Y. Bar-Or, M. Kessel, and M. Shilo, in preparation).

5.6. Role of Hydrophobic Interactions in the Oral Cavity

The oral cavity has several advantages over other host sites in the study of adhesion phenomena: (1) it is more readily accessible than most other host surfaces; (2) it is usually very heavily colonized with an extremely wide range of adherent bacteria; and (3) since the majority of oral microbiota do not flourish outside the oral cavity, the crucial role of adhesion is patently self-evident (Gibbons, 1980).

Microscopic observation of the adherent normal microbiota of the human mouth reveals that each oral surface is colonized by a different pattern of bacteria. For some time, it was believed that adhesion in the oral cavity was mediated by specific lectin-like recognition mechanisms (Gibbons, 1980) and that such specific adhesion correlated to colonization patterns (Gibbons *et al.,* 1976). However, there has been little success in demonstrating such mechanisms [one exception is the lactose-specific coaggregation of oral bacteria (McIntire *et al.,* 1982]. During the past 4 years, the possibility that nonspecific hydrophobic interactions play a major role in oral adhesion has been subjected to extensive experimentation. Some of the main results may be summarized as follows: (1) Oral bacteria are predominantly hydrophobic, including fresh oral isolates from the tooth surface (Weiss *et al.,* 1982), cariogenic laboratory strains (Nesbitt *et al.,* 1982, Olsson and Westergren, 1982; Gibbons and Etherden, 1983), and plaque bacteria obtained and tested directly from the tooth surface (M. Rosenberg *et al.,* 1983c). Some instances in which pronounced cell-surface hydrophobicity was not observed may be due to excessive strain transfer (Westergren and Olsson, 1983) or choice of growth conditions (Rogers *et al.,* 1984). (2) Adhesion to the model tooth surface made up of saliva-coated hydroxyapatite (s-HA) correlates with cell-surface hydrophobicity in various oral strains tested (Nesbitt *et al.,* 1982; Gibbons and Etherden, 1983; Westergren and Olsson, 1983. (3) The inability of nonhydrophobic mutants to adhere to hydrophobic test surfaces, such as hydrocarbons or polystyrene, correlates with their inability to adhere to human teeth (Weiss *et al.,* 1985) or s-HA (Gibbons *et al.,* 1983) compared to the adherent wild-type cells. (4) A hydrophobic fresh isolate of *S. mutans* is retained in the human oral cavity, whereas a nonhydrophobic variant is not (Svanberg *et al.,* 1984). Moreover, the hydrophobic strain proved more cariogenic in laboratory animals than the nonhydrophobic variant (J. Olsson, personal communication). (5) Emulsan, a bacterial amphiphile which blocks adhesion to hydrocarbons, similarly inhibits adhesion of oral and nonoral strains to human buccal epithelial cells and desorbs most of the adherent oral microbiota as well (E. Rosenberg *et al.,* 1983a). (6) Bacteria obtained directly from dental plaque adhere to human buccal epithelial cells *in vitro;* this adhesion is inhibited by the surfactants sodium dodecyl sulfate and Tween 80 (M. Tal, E. Weiss, and M. Rosenberg, unpublished data).

The critical role that saliva probably plays in influencing oral adhesion via hydrophobic interactions is a complex one. On a thermodynamic level, the surface tension of saliva would be expected to influence adhesion to all oral surfaces bathed by it (Busscher *et al.,* 1984). On a molecular level, salivary glycoproteins, antibodies, and other agglutinins can coat the bacterial surface, the substratum, or both, thus affecting adhesion; varying surface tensions and concentrations of such components from subject to subject complicate matters further (Beighton, 1984;

Abbott and Hayes, 1984). In light of such complexities, simpler model systems may prove preferable to s-HA in understanding saliva-mediated effects.

5.7. Adhesion to Other Host Surfaces

Much investigation into adhesion to host tissue has been directed at the gastrointestinal (GI) tract. As in the case of the oral cavity, there is ample evidence to suggest that hydrophobic interactions play a greater role in adhesion than is generally recognized: (1) Certain surfaces in the GI tract are extremely hydrophobic (Hills *et al.,* 1983); moreover, the mucus layer coating intestinal surfaces possesses hydrophobic properties (Smith and LaMont, 1984). (2) A large proportion of hydrophobic bacteria can be found *in situ,* in human fecal samples (Fig. 5) and in the flounder gut (Table II). (3) Adhesion of rough enterobacterial strains to intestinal mucosal cells in greater than that of less hydrophobic, smooth, wild-type cells (Perers *et al.,* 1977; Edebo *et al.,* 1980). Similarly, adhesion of enteropathogenic *Escherichia coli* strains correlates with increased fimbria-mediated hydrophobicity (Smyth *et al.,* 1978; Lindahl *et al.,* 1981; Faris *et al.,* 1981), although there is much disagreement over which organisms are the more hydrophobic (Jann *et al.,* 1981; Ohman *et al.,* 1982; Lindahl *et al.,* 1981). (4) Many laboratory strains of normal intestinal microbiota as well as pathogens that may be associated with the GI tract exhibit cell-surface hydrophobicity. These include strains of *Yersinia enterocolitica* (Faris *et al.,* 1983; Martinez, 1983; Lachica and Zink, 1984a,b; Lachica *et al.,* 1984), *Vibrio cholerae* (Faris *et al.,* 1982; Kabir and Ali, 1983), *Salmonella* (Jiwa and Mansson, 1981; Xiu *et al.,* 1983), *Bifidobacterium* (H. J. M. Oop den Camp, A. Oosterhof, and J. H. Veerkamp, unpublished data), and *Shigella* (Edebo *et al.,* 1983).

Cell-surface hydrophobicity has been reported in a variety of potentially pathogenic bacteria, including *Legionella pneumophila* (Bohach and Snyder, 1983), *Staphylococcus aureus* (M. Rosenberg *et al.,* 1980a; Jonsson and Wadstrom, 1983, 1984; Malmqvist, 1983), *Streptococcus pyogenes* (M. Rosenberg *et al.,* 1981; Ofek *et al.,* 1983; Miorner *et al.,* 1983, 1984; Tylewska *et al.,* 1979), *Mycobacterium lepraemurium* (Katoh and Matsuo, 1983), *Neisseria gonorrhoeae* (Magnusson *et al.,* 1979a,b), and *Chlamydia psittaci* (Vance and Hatch, 1980).

The adhesion of *Lactobacillus fermentum* to the nonsecreting, keratinized part of the stomach in mice was recently shown to correlate with cell-surface hydrophobicity; the presence of erythrosine in the bacterial growth medium reduced hydrophobicity and abolished adhesion to the mouse stomach tissue (P. O. Conway and R. F. Adams, unpublished results).

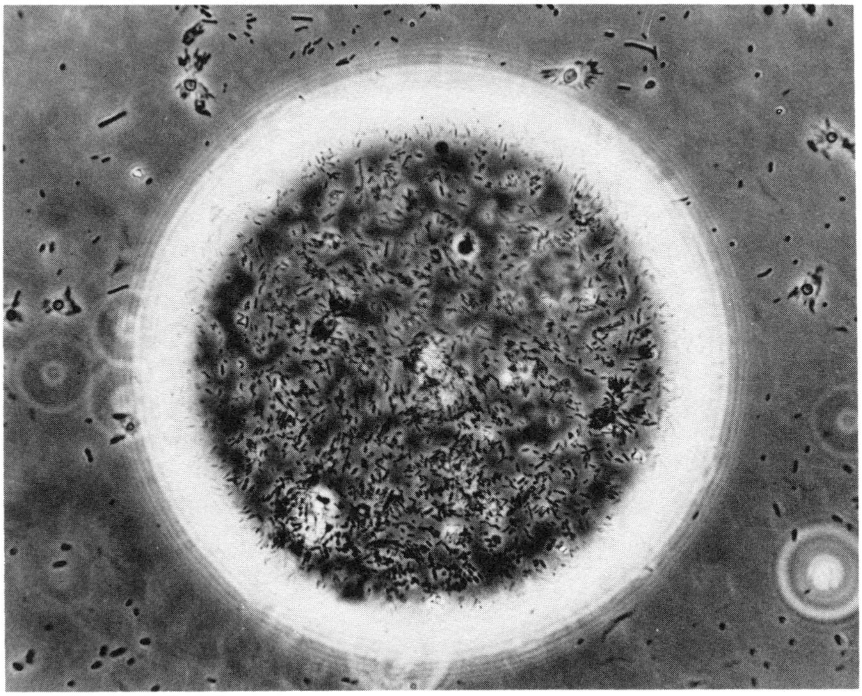

Figure 5. A fresh sample of human feces was washed and suspended in 150 mM phosphate buffer, pH 7.1, to an optical density of 0.9 at 400 nm. Adhesion to 0.2 ml of *n*-hexadecane, *n*-octane, and *p*-xylene was performed as previously described (M. Rosenberg *et al.*, 1983a). Following the mixing, the bottom aqueous phase (1.2 ml) was removed to fresh tubes containing 0.2 ml of the corresponding hydrocarbon, and the phases again mixed. Percent adhesion, as calculated from the drop in turbidity, was: hexadecane, 17%; octane, 45%; xylene, 49%. Shown is an octane droplet with adherent cells, surrounded by aqueous phase, following the mixing procedure. In general, the smaller bacteria tended to adhere at the interface, whereas the larger rods remained in the bulk aqueous phase.

The role of hydrophobic interactions in bacterial adhesion to phagocytic cells has been widely studied in recent years (van Oss, 1978; Absolom *et al.,* 1981, 1982; Edebo *et al.,* 1980); the extent of its participation in relation to more specific mechanisms, however, remains a point of controversy. Hydrophobic interactions play a role in adhesion of *Mycoplasma* to erythrocytes (Banai *et al.,* 1978, 1980; Sobeslavsky *et al.,* 1968), and have probably been overlooked in many cases of bacteria-mediated hemagglutination (R. J. Doyle, personal communication). Similarly, despite the obviously hydrophobic surface properties of human skin and hair, we are not aware of extensive studies on the role of hydrophobic interactions in mediating bacterial adhesion to such surfaces. Do hydrophobic pulmonary surfactants (Cotton and Hills, 1984) mediate or inhibit adhesion to lung tissues? Similarly, what is the role of hydrophobic interactions in mediating adhesion to aging or damaged cells and tissues, e.g., cornea? Is hydrophobicity a factor in adhesion to ear tissue (Hills, 1984a)?

The increasing use of polymeric implants and catheters has led various researchers to study bacterial adhesion to such materials. In many cases, hydrophobic interactions appear to be an important factor (Ashkenazi and Mirelman, 1984; Sugarman, 1982; Hogt *et al.,* 1982, 1983b).

In view of the above, the role of hydrophobic interactions in bacterial pathogenesis is twofold. Cell-surface hydrophobicity may serve to enable initial attachment of bacteria, but may also aid in their removal by phagocytic cells. Ofek *et al.* (1983) have suggested that pathogens may alter surface hydrophobicity as a function of the stage of infection; *S. pyogenes* cells, initially hydrophobic, adhere to and then colonize epithelial cells, but following invasion may become hydrophilic and nonadherent through the elaboration of hyaluronic acid capsule. The observation (Y. Blumberger and M. Rosenberg, unpublished data) that clinical *Serratia* strains become hydrophilic when grown above 37°C is suggestive of a similar example. This phenomenon bears some resemblance to adhesion and desorption of bacteria at marine interfaces (section 5.2).

6. Conclusions

Bacterial adhesion is a multifaceted subject. In some cases (e.g., adhesion of normal human microbiota, cell immobilization, degradation of insoluble pollutants, phagocytosis, nitrogen fixation), adhesion can benefit man. In other situations (colonization of host tissues by pathogens, biofouling) we seek the means to control, inhibit, and desorb adherent microorganisms. Whether trying to promote or prevent bacterial adhesion, an understanding of the underlying mechanisms is of prime importance.

In the present review we have looked at several adhesion phenomena in which hydrophobic interactions appear to play a central role. The possibility exists that the role of hydrophobic interactions in bacterial adhesion is far wider than is generally appreciated. In fact, the participation of hydrophobic interactions in adhesion phenomena is often overlooked, particularly in studies searching for specific (usually sugar-inhibited) adhesion mechanisms. As Kauss and Young (1983) have pointed out, in many investigations a battery of sugars is tested for their ability to inhibit the observed adhesion (if none work, it is often assumed that more exhaustive attempts will eventually uncover the one that does). Only rarely have inhibitors of hydrophobic interactions been compared alongside sugars for their effect on adhesion (e.g., McIntire *et al.,* 1982). Another case in point is the inhibition of adhesion by cell-surface components. It is generally held that the ability of a cell-surface component to inhibit adhesion demonstrates its role as an adhesin in the phenomenon studied. But what if the component is itself an amphipathic molecule, capable of blocking hydrophobic interactions between cell and surface, such as lipopolysaccharide or lipoteichoic acid (Carruthers and Kabat, 1983; Botta, 1981)? In such instances inhibition of adhesion may reflect the participation of hydrophobic interactions rather than a role for the component *per se.* Our appreciation of the extent to which hydrophobic interactions mediate various bacterial adhesion phenomena could be greatly augmented by including agents that interfere with hydrophobic interactions (e.g., surfactants, chaotropic agents) in adhesion assays.

If hydrophobic interactions are, as we submit, chiefly responsible for a great many adhesion phenomena, several corollaries arise. First is the distinct possibility that many bacteria that adhere to surfaces are not too particular about the substrata to which they stick. It can then be argued that the small percentage of cells that do attach to a hospitable surface can quickly proliferate into colonies far outnumbering those that have gone astray. If this is the case, then we must remember when scrutinizing a natural surface that not all the adherent cells we observe may have "meant" to adhere. Similarly, the relative numbers of microorganisms found on a given surface say little about their relative adhesion ability: *A. calcoacaceticus* RAG-1, for example, adheres in much higher numbers to human buccal epithelial cells than does *S. pyogenes,* despite the inability of the former to colonize the oral mucosa (M. Rosenberg *et al.,* 1981; E. Rosenberg *et al.,* 1983a). Similarly, the low relative numbers of filamentous bacteria on buccal epithelial cells as compared with their predominance on the tooth surface (Gibbons, 1980) may be primarily related to their subsequent colonizing ability rather than their initial adhesion. Washed suspensions of filamentous dental plaque bacteria do adhere to buccal epithelial cells *in vitro* in the absence of saliva (M. Tal, E. Weiss, and M. Rosenberg, unpublished results).

Future investigations will be required to deal with the likelihood that the cell-surface hydrophobicity of bacteria *in situ* differs greatly from results obtained using laboratory strains: (1) laboratory strains may lose hydrophobic surface properties upon subculturing; moreover, adherent and aggregating strains, being more difficult to work with in the laboratory, may, over the course of years, have been discarded in favor of less problematic, nonadherent clones; (2) cells in the natural environment may adsorb proteins and other polymers from the surroundings, which in turn affect their surface properties; and (3) bacteria in the environment are often starved; starvation conditions generally result in an increase in hydrophobicity and adhesion.

A problem of increasing interest is the manner in which bacteria modulate cell-surface hydrophobicity in order to detach from, as well as attach to, surfaces. It is clear that bacteria may detach from interfaces as well as adhere to them both in the open environment and in the host. It would be of great interest to learn whether such microorganisms respond to subtle changes in their natural environment which signal them to alter cell-surface hydrophobicity accordingly; in the test tube such changes often appear merely to be related to phase of growth.

How does bacterial hydrophobicity affect other physiological functions? For example, cell-surface hydrophobicity has often been related to the absence of capsule; however, since bacterial capsules may serve to protect the cell from injurious external factors (desiccation, toxic compounds, etc.), the absence of capsule may put the cell at some disadvantage. It has been suggested that cell-surface hydrophobicity is correlated with increased sensitivity to hydrophobic compounds and antibiotics (Miller, 1983; Roantree *et al.,* 1977; Mackey, 1983). However, no correlations between cell-surface hydrophobicity and sensitivity to hydrophobic antibiotics were observed in *S. typhimurium* (Kjelleberg *et al.,* 1985b) or in *A. calcoaceticus* RAG-1 (M. Rosenberg, unpublished data), suggesting that the proposed changes are related to permeability and degree of exposure of outer membrane constituents (Roantree *et al.,* 1977; Leive *et al.,* 1984).

Despite the many techniques available for measuring bacterial hydrophobicity, it is probable that we shall see novel and improved methodology in coming years. Hopefully, these will aid researchers in their attempts to answer the many outstanding questions in this field. One of the most exciting prospects is that the novel genetic techniques that have proven so successful in the study of several *Escherichia coli* adhesion systems will be extended to adhesion phenomena of the type described in this review; some indications of this are already apparent (e.g., Martinez, 1983; Lachica and Zink, 1984a,b; Lachica *et al.,* 1984) in studies on plasmid-mediated hydrophobicity in *Yersinia enterocolitica.*

In closing, we would like to mention another type of close approach that has been mediated by hydrophobic interactions. Ecologically and medically oriented adhesion researchers, once separated by an abyss of different interests and outlooks, have been drawn together by this common mechanism of bacterial adhesion. Hopefully, the synergism resulting from this merger, already evident in many of the investigations we have discussed here, has only just begun.

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References

Abbott, A., and Hayes, M. L., 1984, The conditioning role of saliva in streptococcal attachment to hydroxyapatite surfaces, *J. Gen. Microbiol.* **130**:809–816.

Abraham, S. N., Beachey, E. H., and Simpson, W. A., 1983, Adherence of *Streptococcus pyogenes, Escherichia coli,* and *Pseudomonas aeruginosa* to fibronectin-coated and uncoated epithelial cells, *Infect. Immun.* **41**:1261–1268.

Absolom, D. R., Francis, D. W., Zingg, W., van Oss, C. J., and Neumann, A. W., 1981, Phagocytosis of bacteria by platelets: Surface thermodynamics, *J. Coll. Interface Sci.* **85**:168–177.

Absolom, D. R., van Oss, C. J., Zingg, W., and Neumann, A. W., 1982, Phagocytosis as a surface phenomenon: Opsonization by aspecific adsorption of IgG as a function of bacterial hydrophobicity, *Res. J. Reticuloendothel. Soc.* **31**:59–70.

Absolom, D. R., Lamberti, F. V., Policova, Z., Zingg, W., van Oss, C. J., and Neumann, A. W., 1983, Surface thermodynamics of bacterial adhesion, *Appl. Environ. Microbiol.* **46**:90–97.

Albertsson, P. A., 1958, Particle fractionation in liquid two-phase system. The composition of some phase systems and the behavior of some model particles in them. Application to the isolation of cell walls from microorganisms, *Biochim. Biophys. Acta* **27**:378–395.

Arakawa, K., Tokiwano, K., Ohtomo, N., and Kedaira, H., 1979, A note on the nature of ionic hydrations and hydrophobic interactions in aqueous solutions, *Bull. Chem. Soc. Japan* **52**:2483–2488.

Ashkenazi, S., and Mirelman, D., 1984, Adherence of bacteria to pediatric intravenous catheters and needles and its relation to phlebitis in animals, *Ped. Res.* **18**:1361–1366.

Banai, M., Kahane, I., Razin, S., and Bredt, W., 1978, Adherence of *Mycoplasma gallisepticum* to human erythrocytes, *Infect. Immun.* **21**:365–372.

Banai, M., Razin, S., Bredt, W., and Kahane, I., 1980, Isolation of binding sites to glycophorin from *Mycoplasma pneumoniae* membranes, *Infect. Immun.* **30**:628–634.

Beighton, D., 1984, The influence of saliva on the hydrophobic surface properties of bacteria isolated from oral sites of macaque monkeys, *FEMS Microbiol. Lett.* **21**:239–242.

Ben-Naim, A., 1977, Hydrophobic interaction, in: *l'Eau et les Systemes Biologiques,* Colloques Internationaux de CNRS, no. 246, pp. 215–221, CNRS, Paris.

Bezdek, H. F., and Carlucci, A.F., 1974, Concentration and removal of liquid microlayers from a seawater surface by bursting bubbles, *Limnol. Oceanogr.* **19**:126–132.

Blanchard, D. C., 1983, The production, distribution and bacterial enrichment of the sea-salt aerosol, in: *Air–Sea Exchange of Gases and Particles* (P. S. Liss and W. G. N. Slimm, eds.), pp. 407–454, Reidel, Hingham, Massachusetts.

Blanchard, D. C., and Syzdek, L. D., 1978, Seven problems in bubble and jet drop researches, *Limnol. Oceanogr.* **23**:389–400.

Blanchard, D. C., and Syzdek, L. D., 1982, Water-to-air transfer and enrichment of bacteria in drops from bursting bubbles, *Appl. Environ. Microbiol.* **43**:1001–1005.

Bohach, G. A., and Snyder, I. S., 1983, Characterization of surfaces involved in adherence of *Legionella pneumophila* to *Fischerella* species, *Infect. Immun.* **42**:318–325.

Botta, G. A., 1981, Surface components in adhesion of group A streptococci to pharyngeal epithelial cells, *Curr. Microbiol.* **6**:101–104.

Boyles, W. A., and Lincoln, R. E., 1958, Separation and concentration of bacterial spores and vegetative cells by foam flotation, *Appl. Microbiol.* **6**:327–334.

Breuer, W. V., Ginsburg, H., and Cabantchik, Z. I., 1984, Hydrophobic interactions in *Plasmodium falciparum* invasion into human erythrocytes, *Mol. Biochem. Parasitol.* **12**:125–138.

Bryant, R. D., Costerton, J. W., and Laishley, E. J., 1984, The role of *Thiobacillus albertis* glycocalyx in the adhesion of cells to elemental sulfur, *Can. J. Microbiol.* **30**:81–90.

Busscher, H. J., Weerkamp, A. H., van der Mei, H. C., van Pelt, A. W. J., de Jong, H. P., and Arends, J., 1984, Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion, *Appl. Environ. Microbiol.* **48**:980–983.

Carruthers, M. M., and Kabat, W. J., 1983, Mediation of staphylococcal adherence to mucosal cells by lipoteichoic acid, *Infect. Immun.* **40**:444–446.

Chakravarty, M., Singh, H. D., and Baruah, J. N., 1975, A kinetic model for microbial growth on liquid hydrocarbons. *Biotechnol. Bioeng.* **17**:399–412.

Costerton, J. W., Irvin, R. T., and Cheng, K.-J., 1981, The bacterial glycocalyx in nature and disease, *Annu. Rev. Microbiol.* **351**:299–324.

Cotton, D. B., and Hills, B. A., 1984, Pulmonary surfactant: Hydrophobic nature of the mucosal surface of the human amnion, *J. Physiol.* **349**:411–418.

Courtney, H. S., Simpson, W. A., and Beachey, E. H., 1983, Binding of streptococcal lipoteichoic acid to fatty acid-binding sites on human plasma fibronectin, *J. Bacteriol.* **153**:763–770.

Cover, W. H., and Rittenberg, S. C., 1984, Change in the surface hydrophobicity of substrate cells during bdelloplast formation by *Bdellovibrio bacteriovorus* 109J, *J. Bacteriol.* **157**:391–397.

Cunningham, R. K., Soderstrom, T. O., Gillman, C. F., and van Oss C. J., 1975, Phagocytosis as a surface phenomenon. V. Contact angles and phagocytosis of rough and smooth strains of *Salmonella typhimurium,* and the influence of specific antiserum, *Immunol. Comm.* **4**:429–442.

Dahlback, B., 1983, Marine Microorganisms and Interfaces, Ph.D. thesis, University of Goteborg.

Dahlback, B., Hermansson, M., Kjelleberg, S., Norkrans B., and Pedersen, K., 1980, Cell surface hydrophobicity and charge related to the initial microbial adhesion at the air–water interface, in: *Microbial Adhesion to Surfaces* (R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent, eds.), pp. 540–541, Ellis Horwood, Chichester, England.

Dahlback, B., Hermansson, M., Kjelleberg, S., and Norkrans, B., 1981, The hydrophobicity of bacteria—An important factor in their initial adhesion at the air–water interface, *Arch. Microbiol.* **128**:267–270.

Davison, V. E., and Sanford, B. A., 1982, Factors influencing adherence of *Staphylococcus aureus* to influenza A virus-infected cell cultures, *Infect. Immun.* **37**:946–955.

Dawson, M. P., Humphrey, B. A., and Marshall, K. C., 1981, Adhesion: A tactic in the survival strategy of a marine vibrio during starvation, *Curr. Microbiol.* **6**:195–199.

Dexter, S. C., 1979, Influence of substratum critical surface tension on bacterial adhesion—*In situ* studies, *J. Colloid Interface Sci.* **70**:346–354.

Dexter, S. C., Sullivan, Jr., J. D., Williams III, J., and Watson, S. W., 1975, Influence of substrate wettability on the attachment of marine bacteria to various surfaces, *Appl. Microbiol.* **30**:298–308.

Doyle, R. J., Nedjat-Halem, F., and Singh, J. S., 1984, Hydrophobic characteristics of *Bacillus* spores, *Curr. Microbiol.* **10**:329–332.

Dworkin, M., Keller, K. H., and Weisberg, D., 1983, Experimental observations consistent with a surface tension model of gliding motility of *Myxococcus xanthus, J. Bacteriol.* **155**:1367–1371.

Dyar, M. T., 1948, Electrokinetical studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material and some other surface properties, *J. Bacteriol.* **56**:821–834.

Edebo, L., Kihlstrom, E., Magnusson, K.-E., and Stendahl, O., 1980, The hydrophobic effect and charge effects in the adhesion of enterobacteria to animal cell surfaces and the influences of antibodies of different immunoglobulin classes, in: *Cell Adhesion and Motility* (A. S. G. Curtis and J. D. Pitts, eds.), pp. 65–101, Cambridge University Press, Cambridge.

Edebo, L., Magnusson, K.-E., and Stendahl, O., 1983, Physico-chemical surface properties of *Shigella sonnei, Acta Pathol. Microbiol. Immunol. Scand. B* **91**:101–106.

Ellwood, D. C., Keevil, C. W., Marsh, P. D., Brown, C. M., and Wardell, J. N., 1982, Surface-associated growth, *Phil. Trans. R. Soc. Lond. B* **297**:517–532.

Erne, A. M., Werner, R. G., and Reifenrath, R., 1984, Inhibition of bacterial adhesion by an artificial surfactant, *FEMS Microbiol. Lett.* **23**:205–209.

Faris, A., Wadstrom, T., and Freer, J. H., 1981, Hydrophobic adsorptive and hemagglutinating properties of *Escherichia coli* possessing colonization factor antigens (CFA/I or CFA/II), type 1 pili, or other pili, *Curr. Microbiol.* **5**:67–72.

Faris, A., Lindahl, M., and Wadstrom, T., 1982, High surface hydrophobicity of hemagglutinating *Vibrio cholerae* and other vibrios, *Curr. Microbiol.* **7**:357–362.

Faris, A., Lindahl, M., and Wadstrom, T., 1983, Autoaggregating *Yersinia enterocolitica* express surface fimbriae with high surface hydrophobicity, *J. Appl. Bacteriol.* **55**:97–100.

Fattom, A., and Shilo, M., 1984, Hydrophobicity as an adhesion mechanism of benthic *Cyanobacteria, Appl. Environ. Microbiol.* **47**:135–143.

Ferreiros, C. M., and Criado, M. T., 1984, Expression of surface hydrophobicity encoded by R-plasmids in *Escherichia coli* laboratory strains, *Arch. Microbiol.* **138**:191–194.

Firon, N., Ofek, I., and Sharon, N., 1982, Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli, Biochem. Biophys. Res. Commun.* **105**:1426–1432.

Fletcher, M., 1976, The effects of proteins of bacterial attachment to polystyrene, *J. Gen. Microbiol.* **94**:400–404.

Fletcher, M., 1983, The effects of methanol, ethanol, propanol and butanol on bacterial attachment to surfaces; *J. Gen. Microbiol.* **129**:633–641.

Fletcher, M., 1985, The utilization of glucose by free-living bacteria and those attached to solid surfaces, in: *Abstracts of the 85th Annual Meeting, American Society for Microbiology,* p. 158.

Fletcher, M., and Loeb, G. I., 1979, Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces, *Appl. Environ. Microbiol.* **37**:67–72.

Fletcher, M., and Marshall, K. C., 1982a, Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment, *Appl. Environ. Microbiol.* **44**:184–192.

Fletcher, M., and Marshall, K. C., 1982b, Are solid surfaces of ecological significance to aquatic bacteria?, in: *Advances in Microbial Ecology,* Vol. 6 (K. C. Marshall, ed.), pp. 199–236, Plenum Press, New York.

Gaudin, A. M., Mular, A. L., and O'Connor, R. F., 1960, Separation of microorganisms by flotation. II. Flotation of spores of *Bacillus subtilis* var. *niger, Appl. Microbiol.* **8**:91–97.

Gerson, D. F., and Akit, J., 1980, Cell surface energy, contact angles and phase partition. II. Bacterial cells in biphasic aqueous mixtures, *Biochim. Biophys. Acta* **602**:281–284.

Gibbons, R. J., 1980, Adhesion of bacteria to the surfaces of the mouth, in: *Adsorption of Microorganisms to Surfaces* (R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent, eds.), pp. 351–388, Ellis Horwood, Chichester, England.

Gibbons, R. J., and Etherden, I., 1983, Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles, *Infect. Immun.* **49**:1190–1196.

Gibbons, R. J., Spinell, D. M., and Skobe, Z., 1976, Selective adherence as a determinant of the host tropisms of certain indigenous and pathogenic bacteria, *Infect. Immun.* **13**:238–246.

Gibbons, R. J., Etherden, I., and Skobe, Z., 1983, Association of fimbriae with the hydrophobicity of *Streptococcus sanguis* FC-1 and adherence to salivary pellicles, *Infect. Immun.* **41**:414–417.

Gordon, A. S., and Millero, F. J., 1985, Bacterial respiration and assimilation of organic nutrients sorbed to mineral surfaces, in: *Abstracts of the 85th Annual Meeting, American Society for Microbiology,* p. 278.

Hejkal, T. W., LaRock, P. A., and Winchester, J. W., 1980, Water-to-air fractionation of bacteria, *Appl. Environ. Microbiol.* **39**:335–338.

Hermansson, M., and Dahlback, B., 1983, Bacterial activity at the air/water interface, *Microb. Ecol.* **9**:317–328.

Hermansson, M., and Marshall, K. C., 1985, Utilization of surface localized substrate by non-adhesive marine bacteria, *Microb. Ecol.* **11**:91–105.

Hermansson, M., Kjelleberg, S., and Norkrans, B., 1979, Interaction of pigmented wildtype and pigmentless mutant of *Serratia marcescens* with lipid surface film, *FEMS Microbiol. Lett.* **6**:129–132.

Hermansson, M., Kjelleberg, S., Korhonen, T. K., and Stenstrom, T.-A., 1982, Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion at an air–water surface, *Arch. Microbiol.* **131**:308–312.

Hildebrand, J. H., 1979, Is there a "hydrophobic effect"?, *Proc. Natl. Acad. Sci. USA* **76**:194.

Hill, M. J., James, A. M., and Maxted, W. R., 1963, Some physical investigations of the behaviour of bacterial surfaces. X. The occurrence of lipids in the streptococcal cell wall, *Biochim. Biophys. Acta* **75**:414–424.

Hills, B. A., 1984a, Hydrophobic lining of the eustachian tube imparted by surfactant, *Arch. Otolarynogol.* **110**:779–782.

Hills, B. A., 1984b, Surfactant as a release agent opposing the adhesion of tumor cells in determining malignancy, *Med. Hypotheses* **14**:99–110.

Hills, B. A., Butler, B. F., and Lichtenberger, L. M., 1983, Gastric mucosal barrier: Hydrophobic lining to the lumen of the stomach, *Am. Physiol. Soc.* **1983**:G561–G568.

Hjertén, S., Rosengren, J., and Pahlman, S., 1974, Hydrophobic interaction chromatography. The synthesis and the use of some alkyl and aryl derivatives of agarose, *J. Chromatogr.* **101**:281–288.

Hogt, A. H., Dankert, J., Feijen, J., and de Vries, J. A., 1982, Cell surface hydrophobicity of *Staphylococcus* species and adhesion onto biomaterials, *Antoni van Leeuwenhoek J. Microbiol. Soc.* **48**:496–498.

Hogt, A. H., Dankert, J., and Feijen, J., 1983a, Encapsulation, slime production and surface hydrophobicity of coagulase-negative staphylococci, *FEMS Microbiol. Lett.* **18**:211–215.

Hogt, A. H., Dankert, J., de Vries, J. A., and Feijen, J., 1983b, Adhesion of coagulase-negative staphylococci to biomaterials, *J. Gen. Microbiol.* **129**:2959–2968.

Honda, T., Arita, M., and Miwatani, T., 1984, Characterization of new hydrophobic pili of human enterotoxigenic *Escherichia coli:* A possible new factor, *Infect. Immun.* **43**:959–965.

Humphrey, B. A., Dickson, M. R., and Marshall, K. C., 1979, Physicochemical and *in situ* observations on the adhesion of gliding bacteria to surfaces, *Arch. Microbiol.* **120**:231–238.

Iimura, Y., Hara, S., and Otsuka, K., 1980, Cell surface hydrophobicity as a pellicle formation factor in film strain of *Saccharomyces, Agric. Biol. Chem.* **6**:1215–1222.

Israelachvili, J. N., and Pashley, R. M., 1984, Measurement of the hydrophobic interaction between two hydrophobic surfaces in aqueous electrolyte solutions, *J. Colloid Interface Sci.* **98**:500–514.

Jann, K., Schmidt, G., Blumenstock, E., and Vosbeck, K., 1981, *Escherichia coli* adhesion to *Saccharomyces cerevisiae* and mammalian cells: Role of piliation and surface hydrophobicity, *Infect. Immun.* **32:**484–489.

Jeffrey, W. H., and Paul, J. H., 1985, The effect of attachment to polystyrene on the activity of marine microfouling bacteria, in: *Abstracts of the 85th Annual Meeting, American Society for Microbiology,* p. 229.

Jiwa, S. F. H., and Mansson, I., 1981, Hemagglutinating and hydrophobic surface properties of salmonellae producing enterotoxin neutralized by cholera anti-toxin, *Veterinary Microbiol.* **8:**443–458.

Jones, G. W., and Isaacson, R. E., 1983, Proteinaceous bacterial adhesins and their receptors, *CRC Crit. Rev. Microbiol.* **10:**229–260.

Jonsson, P., and Wadstrom, T., 1983, High surface hydrophobicity of *Staphylococcus aureus* as revealed by hydrophobic interaction chromatography, *Curr. Microbiol.* **8:**347–353.

Jonsson, P., and Wadstrom, T., 1984, Cell surface hydrophobicity of *Staphylococcus aureus* measured by the salt aggregation test (SAT), *Curr. Microbiol.* **10:**203–210.

Kabir, S., and Ali, S., 1983, Characterization of surface properties of *Vibrio cholerae, Infect. Immun.* **39:**1048–1058.

Kappeli, O., and Fiechter, A., 1976, The mode of interaction between the substrate and the cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis, Biotechnol. Bioeng.* **18:**967–974.

Katoh, M., and Matsuo, Y., 1983, Adherence of *Mycobacterium lepraemurium* to tissue culture cells, *Hiroshima J. Med. Sci.* **32:**285–290.

Kauss, H., and Young, D. H., 1983, Fungal spores are agglutinated by proteins and adhere to bean hypocotyls due to nonspecific binding, in: *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins,* pp. 187–196, Alan R. Liss, New York.

Kefford, B., Kjelleberg, S., and Marshall, K. C., 1982, Bacterial scavenging: Utilization of fatty acids localized at a solid–liquid interface, *Arch. Microbiol.* **133:**257–260.

Kennedy, R. S., Finnerty, W. R., Sudarsanan, K., and Young, R. A., 1975, Microbial assimilation of hydrocarbon. I. The fine structure of a hydrocarbon oxidizing *Acinetobacter* sp., *Arch. Microbiol.* **102:**75–83.

Kirschner-Zilber, I., Rosenberg, E., and Gutnick, D., 1980, Incorporation of ^{32}P and growth of pseudomonad UP-2 on *n*-tetracosane, *Appl. Environ. Microbiol.* **40:**1086–1093.

Kitamura, A., 1984, Evidence for an increase in the hydrophobicity of the cell surface during sexual interactions of *Paramecium, Cell Struct. Funct.* **9:**91–95.

Kjelleberg, S., 1984a, Effects of interfaces on survival mechanisms of copiotrophic bacteria in low-nutrient habitats, in: *Current Perspectives in Microbial Ecology* (M. J. Klug and C. A. Reddy, eds.), pp. 151–159, American Society for Microbiology, Washington, D.C.

Kjelleberg, S., 1984b, Adhesion to inanimate surfaces, in: *Microbial Adhesion and Aggregation.* (K. C. Marshall, ed.), pp. 51–70, Springer, Berlin.

Kjelleberg, S., 1985, Mechanisms for bacterial adhesion at gas–liquid interfaces, in: *Bacterial Adhesion: Mechanisms and Physiological Significance* (D. C. Savage and M. M. Fletcher, eds.), pp. 163–194, Plenum Press, New York.

Kjelleberg, S., and Hermansson, M., 1984, Starvation induced effects on bacterial surface characteristics, *Appl. Environ. Microbiol.* **48:**497–503.

Kjelleberg, S., and Stenstrom, T. A., 1980, Lipid surface films: Interaction of bacteria with free fatty acids and phospholipids at the air/water interface, *J. Gen. Microbiol.* **116:**417–423.

Kjelleberg, S., Lagercrantz, C., and Larsson, T., 1980, Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid, *FEMS Microbiol. Lett.* **7:**41–44.

Kjelleberg, S., Humphrey, B. A., and Marshall, K. C., 1982, The effect of interfaces on small starved marine bacteria, *Appl. Environ. Microbiol.* **43:**1166–1172.

Kjelleberg, S., Humphrey, B. A., and Marshall, K. C., 1983, Initial phases of starvation and activity of bacteria at surfaces, *Appl. Environ. Microbiol.* **46:**978–984.

Kjelleberg, S., Marshall, K. C., and Hermansson, M., 1985a, Oligotrophic and copiotrophic marine bacteria—Observations related to attachment, *FEMS Microbiol. Ecol.* (in press).

Kjelleberg, S., Conway, P., and Stenstrom, T. A., 1985b, Inhibition of the starvation survival process of laboratory bacteria strains and the colon microflora of mice, *Arch. Microbiol.* (in press).

Kozel, T. R., 1983, Dissociation of a hydrophobic surface from phagocytosis of encapsulated and non-encapsulated *Cryptococcus neoformans, Infect. Immun.* **39:**1214–1219.

Kupfer, D., and Zusman, D. R., 1984, Changes in cell surface hydrophobicity of *Myxococcus xanthus* are correlated with sporulation-related events in the developmental program, *J. Bacteriol.* **159:**776–779.

Lachica, R. V., and Zink, D. L., 1984a, Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica, Infect. Immun.* **44:**540–543.

Lachica, R. V., and Zink, D. L., 1984b, Determination of plasmid-associated hydrophobicity of *Yersinia enterocolitica* by a latex particle agglutination test, *J. Clin. Microbiol.* **19:**660–663.

Lachica, R. V., Zink, D. L., and Ferris, W. R., 1984, Association of fibril structure formation with cell surface properties of *Yersinia enterocolitica, Infect. Immunol.* **46:**272–275.

Leive, L., Telesetsky, S., Coleman, Jr., W. G. and Carr, D., 1984, Tetracyclines of various hydrophobicities as a probe for permeability of *Escherichia coli* outer membranes, *Antimicrob. Agents Chemother.* **25:**539–544.

Lindahl, M., Faris, A., Wadstrom, T., and Hjerten, S., 1981, A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells, *Biochim. Biophys. Acta* **677:**471–476.

Lis, H., and Sharon, N., 1977, Lectins: Their chemistry and application to immunology, in: *The Antigens* (M. Sela, ed.), Vol. 4, pp. 429–529, Academic Press, New York.

Mackey, B. M., 1983, Changes in antibiotic sensitivity and cell surface hydrophobicity in *Escherichia coli* injured by heating, freezing, drying or gamma radiation, *FEMS Microbiol. Lett.* **20:**395–399.

Magnusson, K.-E., 1980, The hydrophobic effect and how it can be measured with relevance for cell–cell interactions, *Scand. J. Infect. Dis. Suppl.* **24:**131–134.

Magnusson, K.-E., 1982, Hydrophobic interaction—A mechanism of bacterial binding, *Scand. J. Infect. Dis. Suppl.* **33:**32–36.

Magnusson, K.-E., and Johansson, G., 1977, Probing the surface of *Salmonella typhimurium* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers, *FEMS Microbiol. Lett.* **2:**225–228.

Magnusson, K.-E., Kihlstrom, E., Norlander, L., Norqvist, A., Davies, J., and Normark, S., 1979a, Effect of colony type and pH on surface charge and hydrophobicity of *Neisseria gonorrhoeae, Infect. Immun.* **26:**397–401.

Magnusson, K.-E., Kihlstrom, E., Norqvist, A., Davies, J., and Normark, S., 1979b, Effect of iron on surface charge and hydrophobicitiy *Neisseria gonorrhoeae, Infect. Immun.* **26:**402–407.

Malmqvist, T., 1983, Bacterial hydrophobicity measured as partition of palmitic acid between the two immiscible phases of cell surface and buffer, *Acta Pathol. Microbiol. Immunol. Scand. B* **91:**69–73.

Malmqvist, T., Thelestam, M., and Mollby, R., 1984, Hydrophobicity of cultured mammalian cells and some effects of bacterial phospholipases C, *Acta Pathol. Microbiol. Immunol. Scand. B* **92:**127–133.

Marshall, K. C., 1979, Growth at interfaces, in: *Strategies of Microbial Life in Extreme Environments* (M. Shilo, ed.), pp. 281–290, Verlag Chemie, Weinheim.

Marshall, K. C., and Cruickshank, R. H., 1973, Cell surface hydrophobicity and the orientation of certain bacteria at interfaces, *Arch. Mikrobiol.* **91:**29–40.

Marshall, K. C., Cruickshank, R. H., and Bushby, H. V. A., 1975, The orientation of certain root-nodule bacteria at interfaces, including root-hair surfaces, *J. Gen. Microbiol.* **91:**198–200.

Martinez, R. J., 1983, Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica, Infect. Immun.* **41:**921–930.

McIntire, F. C., Crosby, L. K., and Vatter, A. E., 1982, Inhibitors of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34: β-Galactosides, related sugars, and anionic amphipathic compounds, *Infect. Immun.* **36:**371–378.

McLee, A. G., and Davies, S. L., 1972, Linear growth of a *Torulopsis* sp. on *n*-alkanes, *Can. J. Microbiol.* **18:**315–319.

Miller, R. D., 1983, *Legionella pneumophila* cell envelope: Permeability to hydrophobic molecules, *Curr. Microbiol.* **9:**349–354.

Miorner, H., Myhre, E., Bjorck L., and Kronvall, G., 1980, Effect of specific binding of human albumin, fibrinogen, and immunoglobulin G on surface characteristics of bacterial strains as revealed by partition experiments in polymer phase systems, *Infect. Immun.* **29:**879–885.

Miorner, H., Johansson, G., and Kronvall, G., 1983, Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci, *Infect. Immun.* **39:**336–343.

Miorner, H., Havlicek, J., and Kronvall, G., 1984, Surface characteristics of group A streptococci with and without M-protein, *Acta Pathol. Microbiol. Immunol. Scand. B* **92:**23–30.

Miura, Y., Okazaki, M., Hamada, S.-I., Murakawa, S.-I., and Yugen, R., 1977, Assimilation of liquid hydrocarbon by microorganisms. I. Mechanism of hydrocarbon uptake, *Biotechnol. Bioeng.* **19:**701–714.

Moore, R. L., and Marshall, K. C., 1981, Attachment and rosette formation by hyphomicrobia, *Appl. Environ. Microbiol.* **42:**751–757.

Morisaki, H., 1983, Effect of solid–liquid interface on metabolic activity of *Escherichia coli, J. Gen. Appl. Microbiol.* **29:**195–204.

Morisaki, H., 1984, Effect of a liquid–liquid interface on the metabolic activity of *Escherichia coli, J. Gen. Appl. Microbiol.* **30:**35–42.

Morrow, A. W., 1969, Concentration of the virus of foot and mouth disease by foam flotation, *Nature* **222:**489–490.

Mudd, S., and Mudd, E. B. H., 1924a, The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces, *J. Exp. Med.* **40:**633–645.

Mudd, S., and Mudd, E. B. H., 1924b, Certain interfacial tension relations and the behaviour of bacteria in films, *J. Exp. Med.* **40:**647–660.

Nakahara, T., Hisatsuka, K., and Minoda, Y., 1981, Effect of hydrocarbon emulsification on growth and respiration of microorganisms in hydrocarbon media, *J. Ferment. Technol.* **59:**415–418.

Nesbitt, W. E., Doyle, R. J., and Taylor, K. G., 1982, Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite, *Infect. Immun.* **38:**637–644.

Neufeld, R. J., and Zajic, J. E., 1984, The surface activity of *Acinetobacter calcoaceticus* sp. 2CA2, *Biotechnol. Bioeng.* **26:**1108–1113.

Neufeld, R. J., Zajic, J. E., and Gerson, D. F., 1980, Cell surface measurements in hydrocarbon and carbohydrate fermentations, *Appl. Environ. Microbiol.* **39:**511–517.

Neumann, A. W., Absolom, D. R., Francis, D. W., Omenyi, S. N., Spelt, J. K., Policova, Z., Thomson, C., Zingg, W., and van Oss, C. J., 1983, Measurement of surface tensions of blood cells and proteins, in: *Surface Phenomena in Hemotheology:* Their Theoretical, Experimental and Clinical Aspects (A. L. Copley and G. V. F. Seaman, eds.) *Ann. N.Y. Acad. Sci.* **416**:276–298.

Norkrans, B., 1980, Surface microlayers in aquatic environments, in: *Advances in Microbial Ecology,* Vol. 4. (M. Alexander, ed.), pp. 51–85, Plenum Press, New York.

Ochoa, J.-L., 1978, Hydrophobic (interaction) chromatography, *Biochimie* **60**:1–15.

Ofek, I., Whitnack, E., and Beachey, E. H., 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets, *J. Bacteriol.* **154**:139–145.

Ohman, L., 1983, Interaction between *Escherichia coli* Bacteria and Human Granulocytes—The Role of Hydrophobic and Sugarspecific Surface Properties, Ph.D. thesis, University of Linkoping, Sweden.

Ohman, L., Magnusson, K.-E., and Stendahl, O., 1982, The mannose-specific lectin activity of *Escherichia coli* type 1 fimbriae assayed by agglutination of glycolipid-containing liposomes, erythrocytes, and yeast cells and hydrophobic interaction chromatography, *FEMS Microbiol. Lett.* **14**:149–153.

Olsson, J., and Westergren, G., 1982, Hydrophobic surface properties of oral streptococci, *FEMS Microbiol. Lett.* **15**:319–323.

Ouchi, K., and Akiyama, H., 1971, Non-foaming mutants of Sake yeasts: Selection by cell agglutination method and by froth flotation method, *Agric. Biol. Chem.* **7**:1024–1032.

Parker, B. C., Ford, M. A., Gruft, H., and Falinham III, J. O., 1983, Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolization of *Mycobacterium intracellulare* from natural waters, *Am. Rev. Respir. Dis.* **128**:652–656.

Parker, N. D., and Munn, C. B., 1984, Increased cell surface hydrophobicity associated with possession of an additional surface protein by *Aeromonas salmonicida, FEMS Microbiol. Lett.* **21**:233–237.

Pedros-Alio, C., and Brock, T. D., 1983, The importance of attachment to particles for plankonic bacteria, *Arch. Hydrobiol.* **98**:354–379.

Perers, L., Andaker, L., Edebo, L., Stendahl, O., and Tagesson, C., 1977, Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems, *Acta Pathol. Microbiol. Scand. B* **85**:305–316.

Pertsovskaya, A. F., Duda, V. I., and Zvyagintsev, D. G., 1973, Surface ultrastructures of adsorbed microorganisms, *Sov. Soil Sci.* **4**:684–689.

Pines, O., and Gutnick, D., 1984, Alternate hydrophobic sites on the cell surface of *Acinetobacter calcoaceticus* RAG-1, *FEMS Microbiol. Lett.* **22**:307–311.

Pringle, J. H., and Fletcher, M., 1983, Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces, *Appl. Environ. Microbiol.* **45**:811–817.

Pringle, J. H., Fletcher, M., and Ellwood, D. C., 1983, Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition, *J. Gen. Microbiol.* **129**:2557–2559.

Reddy, P. G., Singh, H. D., Roy, P. K., and Baruah, J. N., 1982, Predominant role of hydrocarbon solubilization in the microbial uptake of hydrocarbons, *Biotechnol. Bioeng.* **24**:1241–1269.

Reed, G. B., and Rice, C. E., 1931, The behaviour of acid-fast bacteria in oil and water systems, *J. Bacteriol.* **22**:239–247.

Reuveny, S., Mizrahi, A., Kotler, M., and Freeman, A., 1983, Factors effecting cell attachment, spreading and growth on derivatized microcarriers. II. Introduction of hydrophobic elements, *Biotechnol. Bioeng.* **25**:2969–2980.

Roantree, R. J., Kuo, T.-T., and MacPhee, D. G., 1977, The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium, J. Gen. Microbiol.* **103**:223–234.

Rogers, A. H., Pilowsky, K., and Zilm, P. S., 1984, The effect of growth rate on the adhesion of the oral bacteria *Streptococcus mutans* and *Streptococcus milleri, Arch. Oral Biol.* **29**:147–150.

Romanenko, V. I., 1979, Bacterial growth on slides and electron microscope grids in surface water films and ooze deposits, *Microbiology* **48:**105–109.

Rosenberg, E., Gottlieb, A., and Rosenberg, M., 1983a, Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan, *Infect. Immun.* **39:**1024–1028.

Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M., and Gutnick, D., 1983b, Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbons, *FEMS Microbiol. Lett.* **17:**157–160.

Rosenberg, E., Brown, D. R., and Demain, A. L., 1985, The influence of gramicidin S on hydrophobicity of germinating *Bacillus brevis* spores, *Arch. Microbiol.* **142:**51–54.

Rosenberg, M., 1981, Bacterial adherence to polystyrene: A replica method of screening for bacterial hydrophobicity, *Appl. Environ. Microbiol.* **42:**375–377.

Rosenberg, M., 1982, Adherence of Bacteria to Hydrocarbons and Other Hydrophobic Surfaces, Ph.D. thesis, University of Tel-Aviv, Israel.

Rosenberg, M., 1984a, Bacterial adherence to hydrocarbons: A useful technique for studying cell surface hydrophobicity, *FEMS Microbiol. Lett.* **22:**289–295.

Rosenberg, M., 1984b, Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene, *FEMS Microbiol. Lett.* **25:**41–45.

Rosenberg, M., 1984c, Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity, *J. Bacteriol.* **160:**480–482.

Rosenberg, M., and Rosenberg, E., 1981, Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane, *J. Bacteriol.* **148:**51–57.

Rosenberg, M., Gutnick, D., and Rosenberg, E., 1980a, Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity, *FEMS Microbiol. Lett.* **9:**29–33.

Rosenberg, M., Rosenberg, E., and Gutnick, D., 1980b, Bacterial adherence to hydrocarbons, in: *Microbial Adhesion to Surfaces* (R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent eds.), pp. 541–542, Ellis Horwood, Chichester, England.

Rosenberg, M., Perry, A., Bayer, E. A., Gutnick, D. L., Rosenberg, E., and Ofek, I., 1981, Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane, *Infect. Immun.* **33:**29–33.

Rosenberg, M. Bayer, E. A., Delarea, J., and Rosenberg, E., 1982a, Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane, *Appl. Environ. Microbiol.* **44:**929–937.

Rosenberg, M., Rottem, S., and Rosenberg, E., 1982b, Cell surface hydrophobicity of smooth and rough *Proteus mirabilis* strains as determined by adherence to hydrocarbons, *FEMS Microbiol. Lett.* **13:**167–169.

Rosenberg, M., Rosenberg, E., Judes, H., and Weiss, E., 1983a, Bacterial adherence to hydrocarbons and to surfaces in the oral cavity, *FEMS Microbiol. Lett.* **20:**1–5.

Rosenberg, M., Judes, H., and Weiss, E., 1983b, Desorption of adherent bacteria from a solid hydrophobic surface by oil, *J. Microbiol. Meth.* **1:**239–244.

Rosenberg, M., Judes, H., and Weiss, E., 1983c, Cell surface hydrophobicity of dental plaque microorganisms *in situ, Infect. Immun.* **42:**831–834.

Rubin, A. J., Cassel, E. A., Henderson, O., Johnson, J. D., and Lamb III, J. C., 1966, Microflotation: New low gas-flow rate foam separation technique for bacteria and algae, *Biotechnol. Bioeng.* **8:**135–151.

Runnels, P. L., and Moon, H. W., 1984, Capsule reduces adherence of enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells of pigs, *Infect. Immun.* **45:**737–740.

Rutter, P. R., 1980, The physical chemistry of adhesion of bacteria and other cells, in: *Cell Adhesion and Motility* (A. S. G. Curtis and J. D. Pitts, eds.), pp. 103–135, Cambridge University Press, Cambridge.

Sacks, L. E., and Alderton, G., 1961, Behavior of bacterial spores in aqueous polymer two-phase systems, *J. Bacteriol.* **82:**331–340.

Shields, P. A., and Farrah, S. R., 1983, Influence of salts on electrostatic interactions between poliovirus and membrane filters, *Appl. Environ. Microbiol.* **45:**526–531.

Simpson, W. A., Ofek, I., and Beachey, E. H., 1980, Fatty acid binding sites of serum albumin as membrane receptor analogs for streptococcal lipoteichoic acid, *Infect. Immun.* **29:**119–122.

Smith, B. F., and LaMont, J. T., 1984, Hydrophobic binding properties of bovine gallbladder mucin, *J. Biol. Chem.* **259:**12170–12177.

Smyth, C. J., Jonsson, P., Olsson, E., Soderlind, O., Rosengren, J., Hjerten, S., and Wadstrom, T., 1978, Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography, *Infect. Immun.* **22:**462–472.

Sobel, J. D., and Obedeanu, N., 1984, Role of hydrophobicity in adherence of gram-negative bacteria to epithelial cells, *Ann. Clin. Lab. Sci.* **14:**216–224.

Sobeslavsky, O., Prescott, B., and Chanock, R. M., 1968, Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence, *J. Bacteriol.* **96:**695–705.

Stanley, S. O., and Rose, A. H., 1967, On the clumping of *Corynebacterium xeroxis* as affected by temperature, *J. Gen. Microbiol.* **115:**509–512.

Stenstrom, T.-A., and Kjelleberg, S., 1985, Fimbriae mediated nonspecific adhesion of *Salmonella typhimurium* to mineral particles, *Arch. Microbiol.* (in press).

Sugarman, B., 1982, *In vitro* adherence of bacteria to prosthetic vascular grafts, *Infection* **10:**9–16.

Svanberg, M., Westergren, G., and Olsson, J., 1984, Oral implantation in humans of *Streptococcus mutans* strains with different degrees of hydrophobicity, *Infect. Immun.* **43:**817–821.

Svanborg-Eden, C. S., Bjursten, L.-M., Hull, R., Hull, S. Magnusson, K.-E., Moldovano, Z., and Leffler, H., 1984, Influence of adhesins on the interaction of *Escherichia coli* with human phagocytes, *Infect. Immun.* **44:**672–680.

Syzdek, L. D., 1985, Influence of *Serratia marcescens* pigmentation on cell concentrations in aerosols produced by bursting bubbles, *Appl. Environ. Microbiol.* **49:**173–178.

Tanford, C., 1973, *the Hydrophobic Effect: Formation of Micelles and Biological Membranes,* Wiley-Interscience, New York.

Tanford, C., 1979, Interfacial free energy and the hydrophobic effect, *Proc. Natl. Acad. Sci. USA* **76:**4175–4176.

Todd, I., and Gingell, D., 1980, Red blood cell adhesion. I. Determination of the ionic conditions for adhesion to an oil–water interface, *J. Cell Sci.* **41:**125–133.

Trust, T. J., Kay, W. W., and Ishiguro, E. E., 1983, Cell surface hydrophobicity and macrophage association of *Aeromonas salmonicida, Curr. Microbiol.* **9:**315–318, 1983.

Tylewska, S. K., Hjerten, S., and Wadstrom, T., 1979, Contribution of M protein to the hydrophobic surface properties of *Streptococcus pyogenes, FEMS Microbiol. Lett.* **6:**249–253.

Vance, D. W., and Hatch, T. P., 1980, Surface properties of *Chlamydia psittaci, Infect. Immun.* **29:**175–180.

Van Oss, C. J., 1978, Phagocytosis as a surface phenomenon, *Annu. Rev. Microbiol.* **32:**19–39.

Van Oss, C. J., and Gillman, C. F., 1972, Phagocytosis as a surface phenomenon. I. Contact angles and phagocytosis of non-opsonized bacteria, *Res. J. Reticuloendothel. Soc.* **12:**283–292.

Wadstrom, T., Schmidt, K.-H., Kuhnemund, O., Havlicek, J., and Kohler, W., 1984, Comparative studies on surface hydrophobicity of streptococcal strains of groups A, B, C, D and G, *J. Gen. Microbiol.* **130:**657–664.

Wang, P. Y., 1974, Evidence of hydrophobic interaction in adhesion to tissue, *Nature* **249**:367–368.

Weber, M. E., Blanchard, D. C., and Syzdek, L. D., 1983, The mechanism of scavenging of waterborne bacteria by a rising bubble, *Limnol. Oceanogr.* **28**:101–105.

Weiss, E., Rosenberg, M., Judes, H., and Rosenberg, E., 1982, Cell surface hydrophobicity of adherent oral bacteria, *Curr. Microbiol.* **7**:125–128.

Weiss, E., Judes, H., and Rosenberg, M., 1985, Adherence of a non-oral hydrophobic bacterium to the human tooth surface, *Dental Med.* **3**:11–13.

Wendt, S. L., George, K. L., Parker, B. C., Gruft, H., and Falkinham III, J. O., 1980, Epidemiology of infection by nontuberculous mycobacteria. III. Isolation of potentially pathogenic mycobacteria from aerosols, *Am. Rev. Respir. Dis.* **122**:159–263.

Westergren, G., and Olsson, J., 1983, Hydrophobicity and adherence of oral streptococci after repeated subculture *in vitro, Infect. Immun.* **40**:432–435.

Woodcock, A. H., 1948, Note concerning human respiratory irritation associated with high concentrations of plankton and mass mortality of marine organisms, *J. Mar. Res.* **7**:56–62.

Wyndham, R. C., and Costerton, J. W., 1982, Bacterioneuston involved in the oxidation of hydrocarbons at the air–water interface, *J. Great Lakes Res.* **8**:316–322.

Xiu, J. H., Magnusson, K.-E., Stendahl, O., and Edebo, L., 1983, Physicochemical surface properties and phagocytosis by polymorphonuclear leucocytes of different serogroups of *Salmonella, J. Gen. Microbiol.* **129**:3075–3084.

Young, D. H., and Kauss, H., 1984, Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene, *Appl. Environ. Microbiol.* **47**:616–619.

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Cell Surface Hydrophobicity of Pigmented and Nonpigmented Clinical *Serratia marcescens* Strains

MEL ROSENBERG,[1,2]* YAEL BLUMBERGER,[1] HERBERT JUDES,[1] RONIT BAR-NESS,[1] ETHAN RUBINSTEIN,[3] AND YARDENA MAZOR[1]

School of Dental Medicine[1] and Department of Human Microbiology,[2] Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel 69978, and Chaim Sheba Medical Center, Tel-Hashomer, Israel[3]

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The cell surface hydrophobicity of 10 pigmented and 4 nonpigmented clinical *Serratia marcescens* strains was studied, based on the ability of the strains to adhere to hydrocarbons and to polystyrene. The cell surface hydrophobicity depended greatly on growth temperature; all of the strains tested were adherent following growth at 30°C, whereas none was adherent following growth at 38°C. In previous studies, the pigment prodigiosin has been cited as responsible for cell surface hydrophobicity in various *Serratia* strains. However, the observed ability of the nonpigmented strains to adhere to the test hydrocarbons and to polystyrene indicates that *Serratia* strains can possess hydrophobic surface properties in the absence of this pigment. Moreover, strain 1785 cells were adherent whether they were grown at 30 or 36.5°C, even though pigment was not synthesized at the higher temperature. In *Escherichia coli* correlations have been noted between increased cell surface hydrophobicity and the presence of mannose-specific adhesins; no such relationship was found in the *S. marcescens* strains tested. The expression of cell surface hydrophobicity in clinical *S. marcescens* strains at 30°C and the loss of hydrophobicity at host temperatures raise the possibility that infective cells from the environment are initially hydrophobic, but lose this property upon subsequent proliferation within a host.

In recent years, the role of *Serratia marcescens* strains in causing opportunistic infections has attracted increasing attention (26). Although specific adhesins and aggregating factors of clinical *Serratia marcescens* isolates have been investigated (1, 15, 18), attention has not been given to the cell surface hydrophobicity of such strains, the possible role of such hydrophobicity in microbial pathogenesis, and the molecular components responsible. This is somewhat surprising in view of the many ecologically oriented studies on the importance of the cell surface hydrophobicity of *Serratia* cells in enabling them to adhere at interfaces (21, 22, 24, 24a, 28) and to scavenge nutrients there (12).

Cell surface hydrophobicity is currently regarded as an important factor in mediating bacterial adherence to a wide variety of surfaces (2–6, 8–14, 16, 20–25, 27–29). Most studies on hydrophobicity in *Serratia marcescens* have attributed this property to the presence of the hydrophobic pigment prodigiosin (7, 30, 31) on the cell surface (3, 9, 13, 19, 21, 24). In the present study, we examined the hydrophobic surface properties of pigmented and nonpigmented clinical *Serratia marcescens* isolates based on their ability to adhere to hydrocarbons (24) and to polystyrene (21). Cell surface hydrophobicity was assayed following growth at temperatures which either allow or inhibit prodigiosin synthesis (30, 31). Our data indicate that pigmentation alone cannot account for the hydrophobicity of the strains studied and suggest the possible involvement of additional factor(s). No correlation was found between cell surface hydrophobicity and the presence of mannose-specific adhesins, as has been reported in *Escherichia coli* (15, 18).

The finding that all of the strains tested were hydrophobic following growth at 30°C but not following growth at 38°C raises the possibility that modulation of this property may be of some importance in *Serratia marcescens* pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Clinical *Serratia marcescens* strains were obtained from the Chaim Sheba Medical Center, Tel-Hashomer, Israel (pigmented strains 55, SH-1, 1704, II_9A, BY-1, and I_5 and nonpigmented strains 3255, 577, 2861, and BY-2) or from the Rambam Hospital, Haifa, Israel (pigmented strains 183, 1785, and 1515). *Serratia marcescens* RZ is the strain used in previous investigations (21, 22, 24) and was originally obtained from R. Zack, Tel-Aviv University, Ramat-Aviv, Israel. The bacteria were maintained on brain heart agar (Difco Laboratories, Detroit, Mich.) at 4°C and were transferred every 2 months. For adherence and aggregation experiments, the bacteria from a single colony were spread onto brain heart infusion agar and incubated for 24 h under aerobic conditions at the temperatures indicated below.

Adherence and aggregation experiments. Following growth, the bacteria were scraped from the agar plates, washed twice, suspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, distilled water to 1,000 ml, pH 7.1) to an optical density at 400 nm of 1.0 to 1.2, as measured with a Kontron 710 spectrophotometer (Uvikon, Zurich, Switzerland), and subsequently tested for adherence to hydrocarbons and polystyrene and for mannose-sensitive aggregation of yeast cells. Adherence to hydrocarbons was tested as described previously (24). Briefly, 0.2 ml of a test hydrocarbon (*n*-hexadecane, *n*-octane, or *p*-xylene) was added to 1.2 ml of an aqueous bacterial suspension in standard acid-washed test tubes, and the mixtures were vortexed uniformly for 120 s. Following phase separation, the bottom aqueous phase was carefully removed and transferred to cuvettes, and the optical density at 400 nm was read. Results were expressed as the percent drop in the turbidity of the lower aqueous phase compared with the turbidity of the original suspension. To test for adherence to polystyrene,

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* Corresponding author.

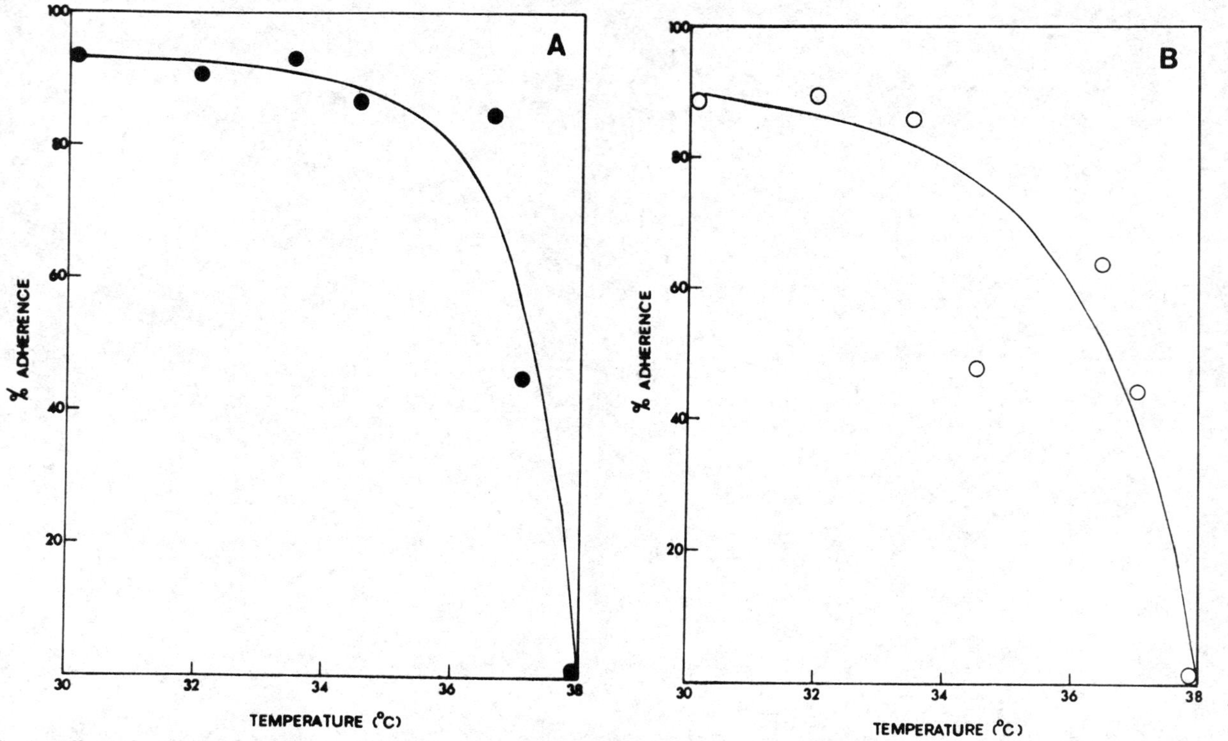

FIG. 1. Effect of growth temperature on adherence to hexadecane. Cells were grown at various temperatures and tested for adherence to 0.2 ml of hexadecane as described in Materials and Methods. The results are presented as the percent adherence of pigmented strain 1785 (A) and nonpigmented strain 3255 (B) to hexadecane as a function of growth temperature. Similar results were obtained for pigmented strain RZ (data not shown).

100-μl portions of a bacterial suspension were placed on polystyrene petri dishes (Miniplast, Ein Shemer, Israel), and following 30 min of incubation at room temperature, each surface was washed vigorously with tap water (21). Adherence was determined visually following staining with Gentian violet (21). The presence of mannose-sensitive adhesins was tested by examining the aggregation of yeast cells, essentially as previously described (15, 16). Dried cells of *Saccharomyces cerevisiae* (Fleischmann's active dry yeast) were suspended in PUM buffer to a concentration of 5 mg/ml. Equal volumes (20 μl) of bacterial and yeast suspensions were mixed on glass slides, and aggregation was scored visually. To ensure that the phenomenon was mannose inhibitable, the assay was simultaneously carried out in the presence of 0.2% (final concentration) D-mannose as a control.

TABLE 1. Adherence characteristics of pigmented *Serratia* strains grown at 30°C

Strain	% Adherence to:[a]			Adherence to polystyrene[b]	Mannose-sensitive aggregation of yeast cells[c]
	n-Hexadecane	*n*-Octane	*p*-Xylene		
183	92	91	90	+	+
55	91	93	88	+	−
1785	94	94	91	+	+
SH-1	78	80	74	+	+
RZ	96	96	95	+	+
1704	84	80	72	+	+
1515	94	85	88	+	+
II$_9$A	89	90	82	+	−
BY-1	92	90	80	+	−
I$_5$	84	85	68	+	−
Mean	89	88	83		

[a] Experiments to determine adherence to hydrocarbons were performed as described in Materials and Methods. The results (means of two separate experiments) are expressed as percent decreases in turbidity compared with the initial cell suspension.

[b] Adherence to polystyrene was scored visually following staining with Gentian violet as described in Materials and Methods.

[c] Aggregation of yeast cells was scored visually as described in Materials and Methods.

RESULTS

Figure 1 shows the effect of growth temperature on the adherence of a pigmented strain and a nonpigmented strain to the test hydrocarbon hexadecane. Both strains were highly adherent following growth at 30°C but became nonadherent as the growth temperature approached 38°C. Of

TABLE 2. Adherence characteristics of nonpigmented *Serratia* strains grown at 30°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
3255	88	90	88	+	−
577	59	62	62	+	+
2861	62	57	55	+	+
BY-2	80	88	76	+	−
Mean	72	74	70		

[a] For experimental conditions see the footnotes to Table 1.

TABLE 3. Adherence characteristics of pigmented *Serratia* strains grown at 38°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
183	0	0	0	–	+
55	0	0	0	–	–
1785	0	1.1	13	–	+
SH-1	0	0	0	–	+
RZ	2.9	2.2	2.3	–	+
1704	0.8	1.4	8.3	–	+
1515	0	0	0.5	–	+
H$_9$A	0	0	3.3	–	–
BY-1	3.4	0.3	1.1	–	–
I$_5$	3.0	3.5	4.0	–	–
Mean	1.0	0.9	3.3		

[a] For experimental conditions (except the growth temperature of cells) see the footnotes to Table 1.

considerable interest was the observation that adherence in the pigmented strain did not correlate directly with pigment synthesis; whereas strain 1785 did not produce pigment when it was grown at temperatures above 36°C, a high proportion (84%) of the cells grown at 36.5°C adhered to hexadecane.

The ability of both pigmented and nonpigmented *Serratia marcescens* strains to adhere to hydrocarbons when they were grown at 30°C is summarized in Tables 1 and 2. The mean level of adherence of the pigmented strains to all three test hydrocarbons (hexadecane, octane, and xylene) (Table 1) was about 15% higher than the level observed for the nonpigmented strains (Table 2). Similarly, all strains grown at 30°C adhered to the hydrophobic solid polystyrene (Tables 1 and 2). In contrast, neither pigmented nor nonpigmented strains adhered to hydrocarbons or to polystyrene when they were grown at 38°C (Tables 3 and 4).

Tables 1 through 4 show the ability of pigmented and nonpigmented isolates to exhibit mannose-sensitive aggregation of yeast cells following growth at 30 or 38°C.

DISCUSSION

The ability of microorganisms to adhere to solid surfaces and to partition at liquid-air and liquid-liquid interfaces is currently regarded as an important factor in bacterial colonization and survival in various habitats (24a). One of the major factors in promoting adherence both in open environments and in hosts is bacterial hydrophobicity. In this context, *Serratia* strains are of considerable interest for the following reasons: (i) they are colonizers of interfaces in the open environment and have also been increasingly implicated in a wide variety of infections in humans (26); and (ii) the avid hydrophobicity observed in pigmented *Serratia* cells has been correlated with their ability to concentrate at air-water interfaces (2–6, 8, 9, 12–14, 28) and water-oil interfaces (22, 24, 24a) and to adhere to solid surfaces (22, 24). Whereas considerable attention has been given to the potential role of *Serratia* hydrophobicity in enabling the colonization of the sea surface by these organisms (2, 6, 9, 14, 28), their dispersion in aerosols and jet bubbles (3–5), and their nutrient scavenging on solid surfaces (12), the possible role of surface hydrophobicity in affecting the infective nature of *Serratia* strains has not been studied.

TABLE 4. Adherence characteristics of nonpigmented *Serratia* strains grown at 38°C[a]

Strain	% Adherence to			Adherence to polystyrene	Mannose-sensitive aggregation of yeast cells
	n-Hexadecane	*n*-Octane	*p*-Xylene		
3255	0	0	0	–	–
577	0	0	5.9	–	+
2861	0	0	2.1	–	+
BY-2	6.2	0	6.4	–	–
Mean	1.6	0	3.6		

[a] For experimental conditions (except the growth temperature) see the footnotes to Table 1.

Our results suggest that most or all clinical *Serratia marcescens* isolates are capable of exhibiting pronounced cell surface hydrophobicity, as determined by adherence to hydrocarbons and polystyrene.

Infectious strains of *Serratia marcescens* may be either pigmented or nonpigmented, with the latter type predominating (7). Since the presence of the pigment prodigiosin has been cited as the factor which is responsible for hydrophobicity in *Serratia* strains (3, 8, 13, 21, 24), we investigated the hydrophobic properties of both pigmented and nonpigmented strains under growth conditions which either allowed or inhibited prodigiosin synthesis. Williams et al. (30, 31) have reported that prodigiosin synthesis occurs at 30°C, but is completely inhibited by growth at higher temperatures. In order to conveniently monitor pigmentation, cells were grown on solid media.

The finding that the mean level of adherence of the pigmented strains was somewhat higher than that of the nonpigmented strains when the organisms were grown at 30°C suggests that the pigment may contribute somewhat to cell surface hydrophobicity. However, the following two lines of evidence indicate that another component(s) is involved: (i) the adherence of all four nonpigmented strains to the test hydrocarbons and to polystyrene following growth at 30°C, and (ii) the observation that strain 1785 remained hydrophobic following growth at 36.5°C, even though pigmentation was no longer visually apparent. These results are consistent with a recent report that a nonpigmented mutant of strain RZ retained partial ability to adhere to hydrocarbons and polystyrene (22).

Preliminary results (data not shown) have shown that a major outer surface protein (molecular weight, approximately 95,000) can be released by shearing (in the presence of 0.2% sodium dodecyl sulfate) from the surfaces of pigmented and nonpigmented strains grown at 30°C, but is absent from the same cells when they are grown at 38°C. The possible contribution of this protein to the cell surface hydrophobicity of pigmented and nonpigmented strains is under investigation.

Several studies on mannose-sensitive adhesins in *E. coli* have suggested that the presence of these adhesins increases cell surface hydrophobicity (11, 27). Since most clinical and other isolates of *Serratia* bear mannose-sensitive adhesins (1, 15, 18), it was of interest to compare cell surface hydrophobicity with mannose-sensitive aggregation of yeast cells. We found no evidence that the ability of clinical *Serratia* isolates to aggregate yeast cells in a mannose-inhibitable fashion is related to the observed hydrophobicity: (i) among the strains that exhibited pronounced cell surface hydrophobicity were strains which did (e.g., strains 183 and

1785 [Table 1]) and did not (e.g., strains 55 and BY-1 [Table 1]) aggregate yeast cells; and (ii) strains which were mannose sensitive following growth at 30°C (Tables 1 and 3) exhibited the same characteristic following growth at 38°C (Tables 2 and 4) despite their complete loss of hydrophobic surface properties following growth at the higher temperature. A separate study has similarly suggested that cell surface hydrophobicity and mannose-specific adherence in *Serratia marcescens* are mutually independent; adherence to hydrocarbons was inhibited by the amphipathic polymer emulsan (20) but not by mannose, whereas adherence of the same bacteria to yeast cells was inhibited by mannose but not by emulsan (M. Rosenberg, A. Gottlieb, and E. Rosenberg, unpublished data).

The finding that all of the clinical isolates tested were hydrophobic following growth at 30°C but lost this property at higher temperatures is of interest. Ofek et al. (17) have implied that pathogenic *Streptococcus pyogenes* cells may regulate their cell surface hydrophobicity, presenting a hydrophobic outer surface in order to promote initial attachment to host tissue, but at some later stage exposing a hydrophilic surface so as to evade phagocytosis (29). Since infections mediated by *Serratia marcescens* often originate in bacteria that are present in the immediate surroundings (26), in which the cells are usually at lower-than-body temperature, the *Serratia marcescens* cells may conceivably be hydrophobic during initial contact with host tissue or with plastic surfaces in contact with a host (10) but not hydrophobic following the onset of infection.

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LITERATURE CITED

1. **Adegbola, R. A., and D. C. Old.** 1982. New fimbrial hemagglutinin in *Serratia* species. Infect. Immun. **38**:306–315.
2. **Bezdek, H. F., and A. F. Carlucci.** 1972. Surface concentration of marine bacteria. Limnol. Oceanogr. **17**:566–569.
3. **Blanchard, D. C., and L. D. Syzdek.** 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. **23**:389–400.
4. **Blanchard, D. C., and L. D. Syzdek.** 1982. Water-to-air transfer and enrichment of bacteria in drops from bursting bubbles. Appl. Environ. Microbiol. **43**:1001–1005.
5. **Blanchard, D. C., L. D. Syzdek, and M. E. Weber.** 1981. Bubble scavenging of bacteria in freshwater quickly produces bacterial enrichment in airborne jet drops. Limnol. Oceanogr. **26**: 961–964.
6. **Carlucci, A. F., and P. M. Williams.** 1965. Concentration of bacteria from sea water by bubble scavenging. J. Cons. Perm. Int. Explor. Mer **30**:28–33.
7. **Ding, M.-J., and R. P. Williams.** 1983. Biosynthesis of prodigiosin by white strains of *Serratia marcescens* isolated from patients. J. Clin. Microbiol. **17**:476–480.
8. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T.-A. Stenstrom.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesin to air-water interfaces. Arch. Microbiol. **131**:308–312.
9. **Hermansson, M., S. Kjelleberg, and B. Norkrans.** 1979. Interaction of pigmented wild type and pigmentless mutant of *Serratia marcescens* with lipid surface film. FEMS Microbiol. Lett. **6**:129–132.
10. **Hogt, A. H., J. Dankert, and J. Feijen.** 1983. Encapsulation, slime production and surface hydrophobicity of coagulase-negative staphylococci. FEMS Microbiol. Lett. **18**:211–215.
11. **Jann, K., G. Schmidt, E. Blumenstock, and K. Vosbeck.** 1981. *Escherichia coli* adhesion to *Saccharomyces cerevisiae* and mammalian cells: role of piliation and surface hydrophobicity. Infect. Immun. **32**:484–489.
12. **Kefford, B., S. Kjelleberg, and K. C. Marshall.** 1982. Bacterial scavenging: utilization of fatty acids localized at a solid-liquid interface. Arch. Microbiol. **133**:257–260.
13. **Kjelleberg, S., C. Lagercrantz, and T. Larsson.** 1980. Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. FEMS Microbiol. Lett. **7**:41–44.
14. **Kjelleberg, S., and T. A. Stenstrom.** 1980. Lipid surface films: interaction of bacteria with free fatty acids and phospholipids at the air/water interface. J. Gen. Microbiol. **116**:417–423.
15. **Mirelman, D., G. Altmann, and Y. Eshdat.** 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. J. Clin. Microbiol. **11**:328–331.
16. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. Infect. Immun. **22**:247–254.
17. **Ofek, I., E. Whitnack, and E. H. Beachey.** 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. **154**:139–145.
18. **Old, D. C., R. Adegbola, and S. S. Scott.** 1983. Multiple fimbrial haemagglutinins in *Serratia* species. Med. Microbiol. Immunol. **172**:107–115.
19. **Purkayastha, M., and R. P. Williams.** 1960. Association of pigment with the cell envelope of *Serratia marcescens* (*Chromobacterium prodigiosum*). Nature (London) **187**:349– 351.
20. **Rosenberg, E., A. Gottlieb, and M. Rosenberg.** 1983. Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. Infect. Immun. **39**:1024–1028.
21. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42**:375–377.
22. **Rosenberg, M.** 1984. Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. J. Bacteriol. **160**:480–482.
23. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. Appl. Environ. Microbiol. **44**:929–937.
24. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.

24a. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. Adv. Microbial Ecol. **9**:353–393.

25. **Rosenberg, M., E. Rosenberg, H. Judes, and E. Weiss.** 1983. Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. FEMS Microbiol. Lett. **20**:1–5.
26. **Sleigh, J. D.** 1983. Antibiotic resistance in *Serratia marcescens*. Br. Med. J. **287**:1651–1653.
27. **Smyth, C. J., P. Jonsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjerten, and T. Wadstrom.** 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. **22**:462–472.
28. **Syzdek, L. D.** 1982. Concentration of *Serratia* in the surface microlayer. Limnol. Oceanogr. **26**:961–964.
29. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32**:19–40.
30. **Williams, R. P.** 1973. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. Appl. Microbiol. **116**: 259–268.
31. **Williams, R. P., C. L. Gott, S. M. H. Qadri, and R. H. Scott.** 1971. Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. J. Bacteriol. **106**:438–443.

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Gutta-percha Solvents—A Comparative Study

A. Tamse, DMD, **U. Unger**, DMD, **Z. Metzger**, DMD, and **M. Rosenberg**, PhD

Gutta-percha solvents are used for both root canal filling and removal of root canal filling materials. A method is presented in which four gutta-percha solvents (chloroform, xylene, Endosolv-E, and orange terpenes) were compared for their effect on three brands of gutta-percha. Chloroform was the most effective solvent for all gutta-percha brands tested. D.M.S. gutta-percha was twice as soluble in chloroform as Hygenic while De-Trey was less soluble than either of the other two brands. Endosolv-E is a relatively efficient solvent for De-Trey gutta-percha.

Gutta-percha solvents are used in a variety of endodontic procedures. These procedures may be grouped as solvent-softened gutta-percha, or customized master cone filling methods or as total removal of root canal fillings for renewal of endodontic treatment and partial removal of a root canal filling while preparing a post space (1). In root canal filling techniques, chloroform has been the solvent of choice due to its high volatility (2–4), whereas in removal techniques either chloroform, xylene, or eucaliptol has been used (5). Chloroform, however, is more effective than the others for zinc oxide-eugenol-based root canal sealers (6).

In recent years, attention has been given to the potential harmful effects that chloroform and xylene may have (7). A possible nondesirable effect of chloroform on periapical tissues (8) is one facet of the issue. The cytotoxic effect of freshly mixed resin chloroform and chloropercha has been demonstrated in cell culture (9) and pronounced tissue reactions have occurred in implantation studies (10, 11). Repeated exposure of endodontists to the solvent vapors may present an environmental risk (12–15). A search for alternative nonharmful but effective solvents of gutta-percha calls for a simple method by which potential solvents may be compared.

A preliminary screening for potentially safe gutta-percha solvents was performed in our laboratory and included various edible solvents and food additives. Among the solvents tested, orange terpenes, a common food additive, had the capacity to dissolve gutta-percha and was, therefore, included in this comparative study.

The purpose of this study was to compare the capacity of solvents to solubilize gutta-percha. Gutta-percha solubility in Endosolv-E and orange terpenes was compared with its solubility in xylene and chloroform.

MATERIALS AND METHODS

Gutta-percha discs (10 mm in diameter, 2-mm thick) were prepared by heating gutta-percha points until a uniform mass was formed and condensing this warm mass into a standard metal mold (16). Discs were prepared using three brands of gutta-percha: Hygenic (Hygenic, Akron, OH), De-Trey (De-Trey, Zurich, Switzerland), and D.M.S. (Dental Mirror Co., Sligo, Republic of Ireland). The average disc weights were 0.41 (±0.02) g, 0.48 (±0.02) g, and 0.36 (±0.2) g, respectively.

One milliliter of solvent (at 37°C) was added to a flat-bottomed preweighted glass vial, each containing a gutta-percha disc. This was followed by mixing on a vortex shaker for 60 s. The solvents containing dissolved gutta-percha were then decanted and the vials containing undissolved gutta-percha were left in a chemical hood for solvent evaporation. Each vial was then weighed at 2, 48, and 72 h to ensure complete evaporation of the solvent; complete evaporation occurred at 48 h as no weight change was recorded in the subsequent 24 h. Vials containing solvent only served as additional controls for complete solvent evaporation. Distilled water was used as a nonsolvent control.

The solvents tested included chloroform (Merck, Darmstadt, West Germany), *p*-xylene (Fluka, Buchs, Switzerland), Endosolv-E (thymol, isoamyl acetate 1,1,1-trichlor; Septodont, Paris, France), and orange terpenes (Hadarim; Hod Hasharon, Israel). Solubilization of gutta-percha was expressed as mean net percentage of loss of weight of the vials containing the samples. All experiments were performed in triplicate, and all vials were coded to ensure objectivity of weighing.

Data within the groups were analyzed by Student's *t* test. Comparison of the groups to each other was carried out by the two-way analysis of variance.

RESULTS

Results of gutta-percha solubilization by the various solvents are presented in Fig. 1. The three brands of gutta-percha tested varied greatly in their solubility, with D.M.S. gutta-percha being more soluble in most solvents than the other brands ($p < 0.05$).

Chloroform dissolved 39.2% (±1.4) of D.M.S. gutta-percha while xylene, Endosolv-E, and orange terpenes dissolved 26.7 (±1.7), 13.9 (±2.3), and 10.5% (±1.2), respectively (Fig. 1). (All differences but those between E and O were significant, $p < 0.001$.) Hygenic gutta-percha was far less soluble, only 19.6% (±2.4) in chloroform, but followed a general pattern similar to that of D.M.S. De-Trey gutta-percha was less soluble than the other two brands, but its solubility in Endosolv-E was greater than in xylene (13.2% (±1.3) as compared with 5.6% (±0.4) ($p < 0.01$)) and was similar to that of chloroform. The distilled water control dissolved none of the gutta-percha brands tested (data not presented).

DISCUSSION

Preliminary screening studies performed with D.M.S. gutta-percha suggested that orange terpenes was a safe solvent of gutta-percha. Our results indicate that it may be an effective agent for slow solubilizing of D.M.S. gutta-percha, with efficiency in a range similar to that of xylene-solubilizing Hygenic gutta-percha. However, it is relatively inefficient in solubilizing either Hygenic or De-Trey gutta-percha.

Differences in solubility of the gutta-percha brands tested resulted most likely from the variation in composition. Gutta-percha resin comprises approximately 17% of the product, while zinc oxide and zinc silicate form most of its weight; some manufacturers include small quantities of waxes in their formula. Changes in content of resin or wax may result in the solubility differences observed. Since gutta-percha manufacturers avoid publishing their formula, it is difficult to come to any conclusions on this matter.

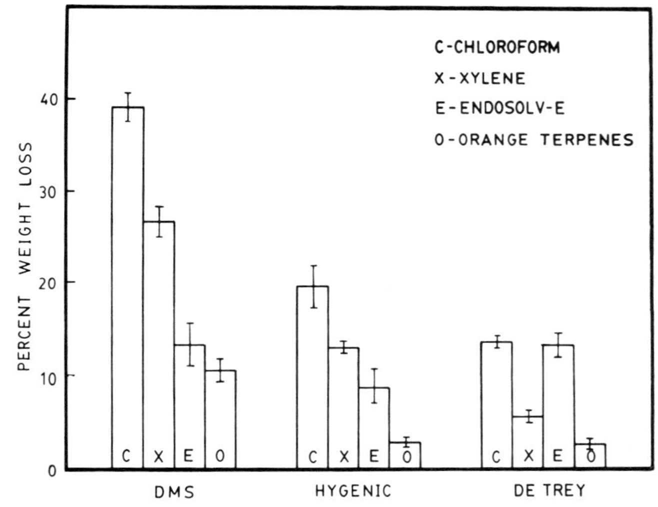

FIG 1. Gutta-percha solubility. Gutta-percha discs were exposed for 60 s to solvents, followed by decantment of solvent and drying for 48 h. Solubility is expressed as percentage of loss weight. Each *bar* represents the mean (±SE) of an experiment performed in triplicate.

The finding that D.M.S. gutta-percha is twice as soluble in chloroform as Hygenic suggests that the former brand may be superior to the latter when used for the preparation of customized master cones by the chloroform dip technique (17). Further studies in this direction are in progress in our laboratory.

SUMMARY

1. A method was described in which gutta-percha solvents were compared.

2. Differences in solubilization of three brands of gutta-percha were demonstrated. D.M.S. gutta-percha is more soluble than Hygenic or De-Trey gutta-percha.

3. Chloroform solubilized gutta-percha more efficiently than any other solvent tested.

4. Endosolv-E was a more effective solvent for De-Trey gutta-percha than xylene.

5. Orange terpenes, a common food additive, may be considered as a solvent for D.M.S. gutta-percha but not for the two other brands tested.

6. The results emphasize the need to test several brands of gutta-percha when testing for solubilization.

This study was performed as a partial fulfillment of the requirements for the DMD degree of U. Unger.

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Dr. Tamse is senior lecturer and coordinator, Section of Endodontology, Dr. Metzger is senior lecturer and director of Research Laboratories, and Dr. Rosenberg is lecturer, Laboratory of Oral Microbiology, School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel. Dr. Unger was affiliated with the School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, and is currently in Kupat-Holim Health Services, Israel.

References

1. Cohen S, Burns RC (eds.). Pathways of the pulp. 1st ed. St. Louis: CB Mosby Co., 1980:147–8, 181–3.

2. Coviello J, Brilliant JD, Wright J. Preliminary scanning electron microscopic study of the chlororesin lateral condensation technqiue. J Endodon 1977;3:54–62.

3. Goldman M. Evaluation of two filling methods for root canals. J Endodon 1975;1:69–72.

4. Larder TC, Prescott AJ, Brayton SM. Gutta percha: a comparative study of three methods of obturation. J Endodon 1976;2:289–94.

5. Ingle J, Beveridge EE. Endodontics. 2nd ed. Philadelphia: Lea & Febiger, 1976:269.

6. Hoover JE. Remington's pharmaceutical sciences. 15th ed. Easton, PA: Mack Publishing Co., 1975.

7. Spangberg L, Engstrom B. Studies on root canal medicaments. II. Cytotoxic effect of medicaments used in root canal filling. Acta Odontol Scand 1967;35:183–6.

8. Spangberg L, Langeland K. Biologic effects of dental material. I. Toxicity of root canal filling materials on HeLa cells *in vitro*. Oral Surg 1973;35:402–14.

9. Wennberg, A. Biological evaluation of root canal sealers using in vitro

and in vivo methods. J Endodon 1980;6:784–7.

10. Olsson B, Sliwkowski A, Langeland K. Intra-osseous implantation for biological evaluation of endodontic materials. J Endodon 1981;7:253–65.

11. Olsson B, Wennberg A. Early tissue reaction to endodontic filling materials. Endodon Dent Traumatol 1985;1:138–41.

12. Gusev IS. Reflective effects of microconcentrations of benzene, toluene, xylene and their comparative assessment. Hyg Sanit 1965;30:331–6.

13. Goldine I. Can xylene (xylol) provoke compulsive seizures? Ind Med Surg 1960;29:33–5.

14. Challen PJR, Hickish DE, Bedford J. Chronic chloroform intoxication. Br J Ind Med 1958;15:243–9.

15. Chloroform (trichlorometane) hygenic guide series. Am Ind Hyg Assoc J 1965;26:636–9.

16. ANSI/ADA specification no. 57 for endodontic materials. 1980.

17. Weine FS. Endodontic therapy. 2nd ed. St. Louis: CV Mosby Co., 1976:248–50.

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The QuadLoop: a novel device for plating and streaking microbial samples

Mel Rosenberg[a,b,*] and Ervin Weiss[b]

[a]*Department of Human Microbiology and* [b]*Maurice and Gabriela Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978 (Israel)*

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Summary

A novel disposable plastic device which facilitates microbial sampling and dilution streaking procedures is described. The basic features of the device are (i) a sphere of ca. 4 mm diameter, on which the microbial sample is taken up, which is attached to (ii) a square handle which enables the device to be held in four distinct positions. The microbial sample is taken up on one quadrant of the sphere and plated. Subsequent 90° turns of the device are then used to dilute the inoculum on the agar plate for colony isolation, thus eliminating the need to employ several disposable loops for a single sampling and streaking procedure. The device can be used to sample colonies, liquids and solid surfaces.

Key words: *Colony isolation – Dilution – Inoculation – Loop – Sampling – Streaking*

Description

Colony isolation is a basic requirement of most, if not all, microbiological laboratories. In order to dilute inocula so that individual clones can be observed, samples are successively diluted on agar plates by the process known as 'dilution streaking'. Until recently, this procedure was generally performed using wire loops which must be flamed and cooled several times in order to streak a single plate for colony isolation. In order to eliminate these tedious steps and possible concomitant aerosol formation, disposable polystyrene loops were introduced. The main disadvantage of such loops is that several are generally necessary to obtain and plate the sample, and then successively dilute it over the agar plate surface. The development of a device which singlehandedly enables sampling and subsequent dilution streakings is thus of potential benefit. Indeed, the patent literature [1 – 3] contains several innovative approaches to this problem. Some of these are illustrated in Fig. 1. However, to the best of our knowledge, none have been employed on a wide scale.

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[*] To whom correspondence should be addressed at the Maurice and Gabriela Goldschleger School of Dental Medicine.

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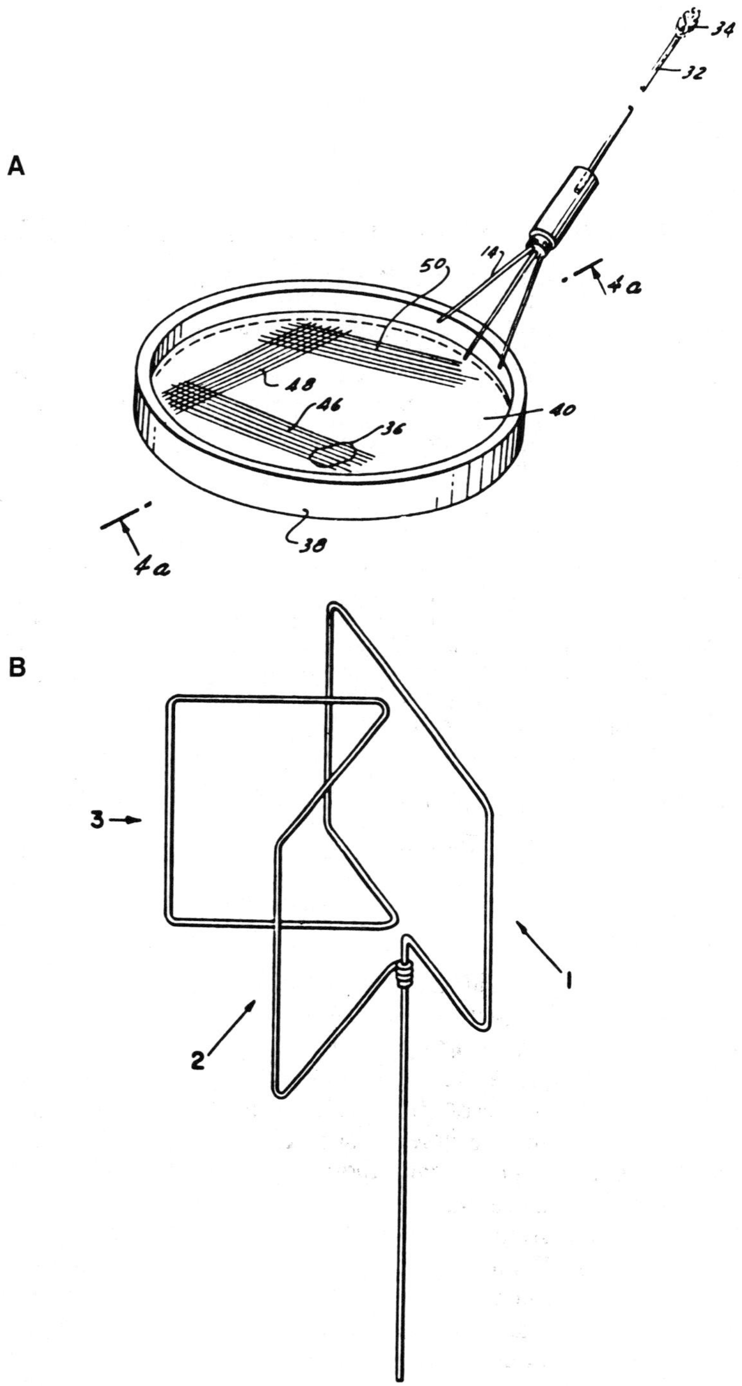
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Fig. 1. Innovative designs for bacteriological transfer loops. Pardos [1] described a transfer and inoculating loop having several circumferentially spaced fingers (Fig. 1A). A three plane instrument was described by Vacanti [2] (Fig. 1B).

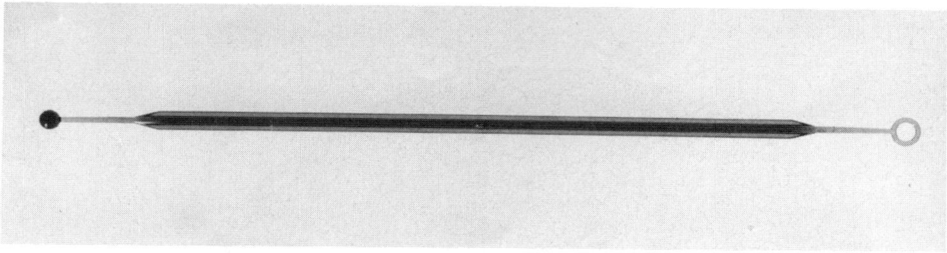

Fig. 2. Photograph of QuadLoop prototype. The sampling and streaking sphere is attached to a square handle. A 10 μl loop is provided at the opposite end for sampling fixed volumes.

Studies in this laboratory have led to development of a user-friendly device, the 'QuadLoop'. The QuadLoop enables sampling and dilution streaking to be performed almost simultaneously, using a single device. The basic design of the QuadLoop is shown in Fig. 2. The innovative features of the device include (i) a

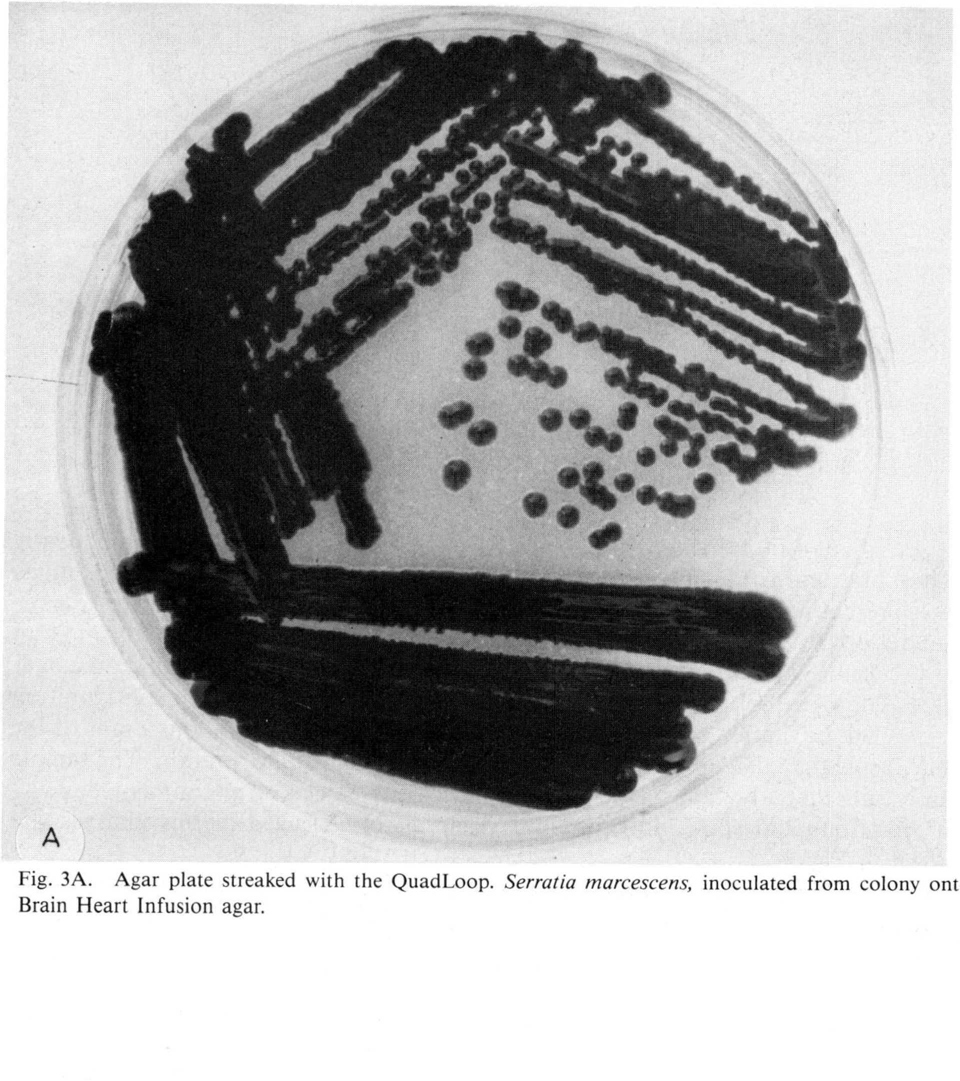

Fig. 3A. Agar plate streaked with the QuadLoop. *Serratia marcescens,* inoculated from colony onto Brain Heart Infusion agar.

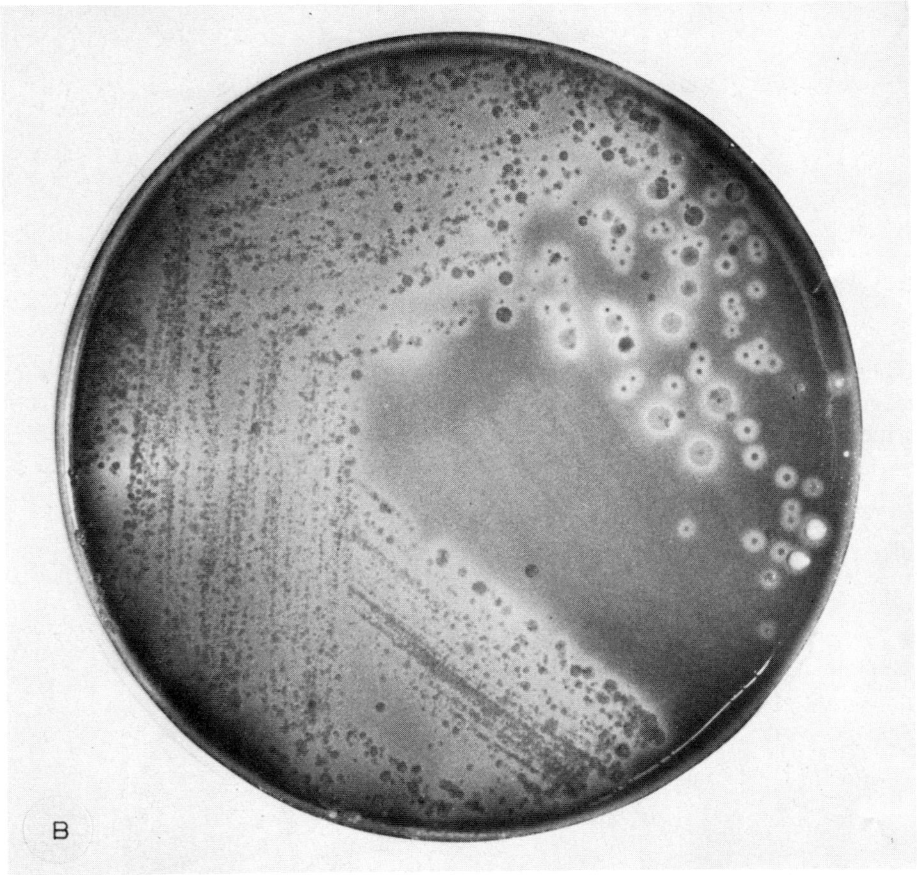

Fig. 3B. Agar plates streaked using the QuadLoop. Saliva sample, inoculated onto tryptic soy agar containing 5% sheep blood.

sphere of ca. 4 mm diameter, on which the inoculum is obtained and subsequently plated out, and (ii) a square handle which defines four distinct holding positions, each presenting a distinct surface on the sphere itself. Thus, in order to use the QuadLoop, it is held as one would hold a regular disposable loop. The sample to be streaked (e.g., colony) is touched and then spread over one quadrant of the agar plate. The QuadLoop is then rotated in hand to the adjacent handle configuration (i.e., by 90°). Dilution streaking is then carried out onto the second quadrant of the plate, using the fresh area of the sphere obtained by the handle rotation. The sample can be subsequently diluted twice more, by successive clockwise or counter-clockwise 90° rotations and streaking procedure. Agar plates, streaked in this manner, are shown in Fig. 3.

We have employed the QuadLoop to transfer a wide variety of microbial colonies, including strains of *Escherichia coli, Staphyloccus aureus* and *S. epidermidis,* *Acinetobacter calcoaceticus, Pseudomonas aeruginosa* and *P. fluorescens, Bacillus cereus, Serratia marcescens, Micrococcus luteus, Streptomyces clavigularis* and *Streptomyces satsumaensis, Streptococcus pyogenes* and *Streptococcus mutans, Ustilago maydis* and *Saccharomyces cerevisiae.* In all cases, colony isolation was comparable to that obtained using standard methods (wire loop and disposable 10 μl loops), but could be carried out in less than one-half the time, and without occasional gouging of the agar surface, even at agar concentrations as low as 1%.

In addition to its ability to transfer colonies and other samples obtained from solid and semi-solid surfaces, the QuadLoop can also singlehandedly sample and streak liquids for colony isolation (Fig. 3). We have found that the simplest way to do this is to touch the sphere to the liquid surface. In this manner, approximately 2 μl are taken up onto one quadrant of the sphere, and the other three faces can be used for subsequent dilution streakings for colony isolation as described above. We have found such a procedure to be extremely convenient for urine determinations, as well as sampling and plating of milk products and other beverages.

Acknowledgements

We thank Miniplast, Ein Shemer, Israel, for supplying prototype QuadLoop samples, and Ronit Bar-Ness, Yardena Mazor and Miriam Shaharbany for excellent technical assistance.

References

1 Pardos, G. (1977) Bacteriological transfer loop. U.S. Patent 4,010,077.

2 Vacanti, M.F. (1978) Device for plating and streaking a microbiological sample. U.S. Patent 4, 102,748.

3 Connors, J.J. (1964) Bacteriological transfer loops. U.S. Patent 3,147,197.

PREPARATIVE BIOCHEMISTRY, 16(2), 133-141 (1986)

SEPARATION OF AMPHIPATHIC PROTEINS BASED ON ADSORPTION TO HEXADECANE:WATER INTERFACES

Mel Rosenberg[1,2], Zinaida Zosim[3], Naomi Regimov[1]
and Ilana Eli[1]

School of Dental Medicine[1] and Department of Human
Microbiology[2], Sackler Faculty of Medicine, and
Department of Microbiology, George S. Wise Faculty of Life
Sciences[3], Tel-Aviv University, Ramat-Aviv, Israel 69978.

ABSTRACT

A method is proposed for the relatively simple and rapid separation of amphipathic biopolymers, based on adsorption onto and desorption from the surface of hexadecane droplets. Adsorption to the hexadecane:water interface was carried out by mixing hexadecane with aqueous protein solutions at room temperature. Desorption was performed by consecutive solidification and melting of the liquid hydrocarbon (m.p. 18° C), resulting in coalescence of the droplets and reappearance of the desorbed moiety in the bulk aqueous phase. Of interest was the observation that lysozyme remains enzymatically active following this procedure.

INTRODUCTION

The separation of amphipathic proteins and other biopolymers on the basis of their relative hydrophobic

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properties is of fundamental and applied interest. The main techniques currently used are based on chromatography employing solid hydrophobic phases such as n-alkyl and aryl agaroses (1) and n-alkyl-bonded silica supports (2). Following adsorption of the amphipathic moieties to the solid support, elution may be carried out by decreasing salt gradients, or increasing the concentrations of detergents or organic solvents. Problems which may be encountered with such an approach include denaturation, and the expense of processing large quantities of material. In the present manuscript we propose an alternative procedure, based on (i) adsorption of amphipathic proteins onto liquid hydrocarbon:water interfaces, following dispersion of the hydrocarbon, and (ii) desorption from the water:hydrocarbon interface by solidification and melting of the hydrocarbon phase. The hydrocarbon employed, hexadecane, has a melting point of 18°C.

MATERIALS AND METHODS

Extraction of a Standard Protein Mixture

The standard protein mixture used was the molecular weight markers kit, Dalton Mark VII-L (Sigma, St. Louis, MO), containing seven proteins, of the following approximate molecular masses, in kD: bovine albumin, 66; ovalbumin, 45 ; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 36 (subunit); carbonic anhydrase from bovine erythrocytes, 29; treated trypsinogen from bovine pancreas, 24; trypsin inhibitor from soybean, 20.1; α-lactalbumin, 14.2. The mixture was dissolved in distilled water to a final concentration of 1 mg/ml total protein. To one ml of aqueous protein suspension in an acid-washed, round bottom test tube, 0.5 ml hexadecane was added, and the mixture vortexed for 2 min on a Thermolyne Maxi Mix (Sybron, Dubuque, Iowa) at room temperature (20° ± 2°C). Control test tubes (no hexadecane added) were similarly treated. Following phase separation, the lower aqueous phase was removed carefully using a Pasteur pipette; one ml of distilled water was added to the test tube and the contents equilibrated at 4 C. Following solidification of the hexadecane layer, the test tube was allowed to equilibrate at room temperature (20° ± 2° C) and the aqueous phase again removed. Aliquots of the aqueous phase prior to and following the hexadecane solidification and melting procedure were analyzed on a 15% SDS-polyacrylamide gel, as described by Laemmli (3).

Extraction of Lysozyme from Human Saliva

Unstimulated , whole saliva was collected from one donor, and clarified by centifugation at 12,000 g for 10 min. To 1 ml of clarified saliva 0.5 ml of hexadecane was added and the mixture vortexed for 2 min at room temperature. Following phase separation the lower aqueous phase was removed. The hexadecane layer was washed with 1.0 ml PBS (phosphate buffered saline: 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and distilled water to one liter, pH 7.2), which was subsequently removed and replaced with 1.0 ml fresh PBS. Contents of the test tube were then equilibrated at 4°C in order to solidify the hexadecane layer; the test tube was then allowed to equilibrate with room temperature, with concomitant melting of the hexadecane.

In order to assay lysozyme activity, 25 µl aliquots were applied in quadruplicate to paper pads (Schleicher and Schull, West Germany, no. 321260). Following drying, the pads were placed on lawns of Micrococcus lysodeikticus (ATCC 4698) spread on nutrient agar plates. Following 18 h incubation at 30 C the diameters of the inhibition zones were compared with those obtained using known salivary dilutions, and the relative activity obtained by interpolation.

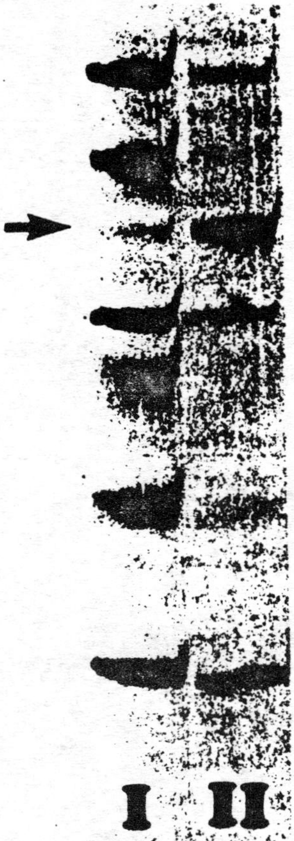

FIGURE 1
SDS-Polyacrylamide gel electrophoresis of seven marker proteins. Mixing of seven marker proteins in the presence of hexadecane, as described in Materials and Methods brought about an almost complete loss of glyceraldehyde-3 phosphate dehydrogenase (G3PD, arrow; lane I) which could be eluted by the solidification and melting procedure (lane II). Marker proteins employed, were (top to bottom): bovine albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibititor, and α-lactalbumin.

Extraction of Lysozyme from PBS

Lysozyme (Sigma, St. Louis, MO, no. L6876, from chicken egg white) was dissolved in 1 ml PBS to a final concentration of 200 ug/ml and used fresh. To one ml of solution, 0.5 ml hexadecane was added and the mixture vortexed three times for two minute durations. The lower phase was immediately removed and 1 ml of distilled water added to the hexadecane. Desorption was carried out as described above. Twenty-five µl aliquots of the liquid removed prior to and following the solidification/melting procedure were assayed for enzyme activity as described above. Relative activity was calculated from two calibration curves obtained using various lysozyme concentrations, dissolved in water or PBS, respectively.

RESULTS

When a commercial preparation of a mixture of seven molecular weight marker proteins was vortexed in the presence of hexadecane one of the bands, corresponding to glyceraldehyde-3-phosphate dehydrogenase (G3PD), was almost completely removed from the aqueous phase, as observed by SDS-polyacrylamide electrophoresis of the mixture (Figure 1, lane 1). When the bottom aqueous phase was removed and replaced, and the hexadecane layer subsequently solidified and melted, the enzyme was eluted (Figure 1, lane 2).

The mixing of whole, clarified saliva in the presence of hexadecane brought about a 70% loss in lysozyme activity, as compared with that prior to mixing (Table 1 and Fig. 2). Forty-two percent of the activity lost in the mixing procedure (i.e., 30% of the original activity of the sample) could be recovered from the hexadecane:water interface following phase transition of the hydrocarbon.

Lysozyme could be similarly extracted and eluted from PBS (Table 2). Seventy percent of the activity removed from the

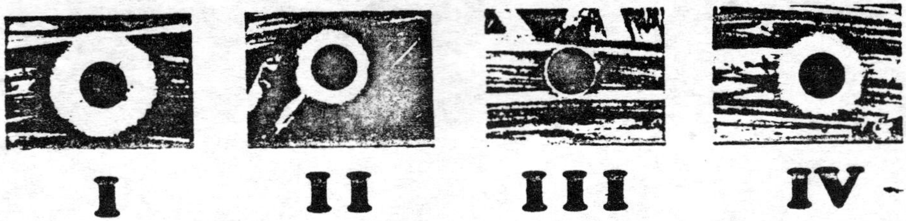

FIGURE 2
Adsorption and desorption of lysozyme from clarified human saliva. Lysozyme activity was assayed on lawns of M. lysodeikticus as described in Materials and Methods. (I) Zone of inhibition of clarified human saliva; (II) Loss of activity following mixing with hexadecane; (III) No significant activity observed in PBS wash of hexadecane layer; (IV) Reappearance of activity in aqueous phase following solidification and melting of hexadecane layer.

TABLE I
Adsorption and Release of Lysozyme from Clarified Human Saliva.[a]

Sample	Relative Activity[b]
Unextracted clarified saliva	1.00
Following mixing with hexadecane	0.30
Activity adsorbed to interface following Mixing procedure[c]	0.70
Aqueous sample following solidification and melting of hexadecane layer	0.30

[a] Lysozyme activity was measured by applying 25 µl aliquots to paper pads, and measuring their zones of inhibition on lawns of M. lysodeikticus as described in Materials and Methods.

[b] Calculated from calibration curve using various salivary dilutions as described in Materials and Methods.

[c] i.e., loss in activity following mixing, presumably due to adsorption to the interface.

TABLE II
Adsorption and Desorption of Lysozyme from PBS.[a]

Sample	Relative Activity[b]
Solution prior to mixing with hexadecane	1.00[b]
Solution following mixing with hexadecane	0.56[b]
Activity adsorbed to interface following mixing procedure[c]	0.44
Aqueous sample following solidification and melting of hexadecane layer	0.31[d]

[a] Lysozyme activity was measured by applying aliquots to paper pads, and measuring their zones of inhibition on lawns of M. lysodeikticus as described in Materials and Methods.

[b] Calculated from calibration curve using various known lysozyme concentrations in PBS.

[c] i.e., loss of activity in aqueous phase following mixing, presumably due to adsorption to interface

[d] Calculated from calibration curve using known lysozyme concentrations in distilled water.

PBS upon vortexing could be recovered from the distilled water following solidification and melting of the hydrocarbon. Elution into PBS, however, was less effective (not shown).

DISCUSSION

In recent years, adsorption to liquid hydrocarbon droplets has been extensively used to compare the relative hydrophobicity of various microbial cells (4,5), and to separate hydrophobic and nonhydrophobic bacteria (5,6). Of interest was the finding (7) that solidification and melting of the liquid hydrocarbon causes coalescence of the droplets and concomitant desorption of bound cells into the bulk aqueous phase. This observation raised the possibility that individual molecules may behave in a similar manner.

The results presented here suggest that certain proteins from commercial and biological mixtures can be removed to the aqueous:hexadecane interface following a simple mixing procedure. Moreover, the desorbed proteins can be eluted by solidifying and melting hydrocarbon phase, concomitant with droplet coalescence. The finding that up to 70% of the lysozyme activity adsorbed to the aqueous:hexadecane interface could be recovered using this procedure suggests its potential for enzyme separation and purification.

The underlying mechanism of desorption of proteins from the water:hexadecane interface following phase transition of the hydrocarbon appears to be related to the vast decrease in interfacial surface area upon solidification and coalescence of the hydrocarbon droplets. It has previously been suggested that the desorption of bound microbial cells is related to their deformation of the interface, and that this deformation is perturbed upon solidification (Paul Rutter, personal communication).

Additional possible uses of this technique include investigations into the role of amphipathic biopolymers in various biological phenomena. For example, it was recently shown that extraction of saliva with hexadecane removes its ability to inhibit bacterial adhesion to polystyrene (8). Solidification and melting of the hexadecane phase resulted in partial reappearance of the observed inhibition, suggesting that the inhibitory amphipathic molecules involved can be isolated and studied (I. Eli and M. Rosenberg, unpublished data).

The proposed approach may have advantages of potential use in commercial purification techniques: (i) the availability and low cost of hexadecane and other suitable hydrocarbons, as compared with hydrophobic chromatographic supports; (ii) it can be performed relatively rapidly, and (iii) desorption does not require addition of eluents. It is clear that for specific separation purposes, various aspects of the procedure may be optimized, e.g., mixing energy and duration, composition of the aqueous and hydrocarbon phases, and so on.

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REFERENCES

1. S. Shaltiel, Meth. Enzymol., 104, 69-96 (1984).
2. N.T. Miller, B. Feibush, K. Corina, S. Powers-Lee and B.L. Karger, Anal. Biochem., 148, 510-517 (1985).
3. U.K. Laemmli, Nature, 227, 1 (1971).
4. M. Rosenberg, D. Gutnick and E. Rosenberg, FEMS Microbiol. Lett., 9, 29-33 (1980).
5. M. Rosenberg, FEMS Microbiol. Lett. 22, 289-295 (1984).
6. M. Rosenberg and E. Rosenberg, J. Bacteriol. 148, 51-57 (1981).
7. M. Rosenberg, Ph.D. Thesis, Tel-Aviv University (1982).
8. I. Eli, N. Regimov and M. Rosenberg, submitted for publication.

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Measurement of the kinetics of bacterial adherence to hexadecane in polystyrene cuvettes

(Hexadecane; adherence; *Streptococcus pyogenes*; *Acinetobacter calcoaceticus*; *Serratia marcescens*)

Danny Sharon [a], Ronit Bar-Ness [a] and Mel Rosenberg [a,b,*]

[a] *School of Dental Medicine and* [b] *Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University Ramat-Aviv, 69978 Tel-Aviv, Israel*

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1. SUMMARY

The kinetics of bacterial adherence to hexadecane were measured using disposable polystyrene cuvettes. The rate of adherence was exponential, and was itself linearly dependent on the water : hexadecane ratio employed. The dependency of the rate of adherence on the water : hexadecane ratio, termed the removal coefficient, varied from 4.7 min^{-1} for *Streptococcus pyogenes* to 72 min^{-1} for *Acinetobacter calcoaceticus.* The removal coefficient of *Serratia marcescens* was a function of growth temperature, 48 min^{-1} following growth at 30°C as opposed to 5.8 min^{-1} for 35°C-grown cells.

2. INTRODUCTION

Bacterial adherence to hydrocarbons is a simple, inexpensive and rapid method for investigating the hydrophobic surface properties of bacterial cells [1]. However, until recently, the potential usefulness of this technique was limited by its semi-quantitative nature [2]. Recently, Lichtenberg et al. [3] proposed a kinetic approach to bacterial adherence to hexadecane based on the rate, rather than the extent of adherence. In this approach, the rates of adherence are measured in a series of test tubes containing different hydrocarbon volumes. The dependence of the adherence rate on the hydrocarbon : water ratio was found to be linear. The slope, referred to as the 'removal coefficient' [4], was proposed as a quantitative index of cell surface hydrophobicity [3].

Until now, the adherence assay itself has been performed in test tubes. Following the mixing of aqueous bacterial suspension with the test hydrocarbon, the bottom phase is either removed for spectrophotometric reading to a cuvette [1], or preferably read directly in the test tube [3], to determine the fraction of adherent cells bound to the floating hydrocarbon droplets. Since the rate and extent of adherence are affected by the size of the test tube (unpublished data), standardization of the technique would require use of a standard vessel [2]; each brand of photometer, however, accepts test tubes of different dimensions. In order to overcome this difficulty, we have attempted to combine mixing and reading procedures using square, disposable, polystyrene cuvettes which fit

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* To whom correspondence should be addressed, at the School of Dental Medicine.

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practically all spectrophotometers. Despite the square dimensions, the semi-micro cuvettes could easily be vortexed and the kinetics of adherence measured in a manner which can be repeated in various laboratories. In the present report, we have used this modification to study the kinetics of bacterial adherence to hexadecane. The cells employed were *A. calcoaceticus* HO1-N [5], *S. pyogenes* M-5 [5,6], and *S. marcescens* 3255 [7] following growth at two different temperatures.

3. MATERIALS AND METHODS

In order to study adherence, bacterial strains were inoculated from agar plates into brain heart infusion broth (Difco) and, following growth, were inoculated 1 : 200 into liquid medium of the same composition, and grown to stationary phase (18 h) with shaking, at the indicated temperature. Bacteria were washed twice and suspended in phosphate buffer (22.2 g K_2HPO_4 $3H_2O$, 7.26 g HK_2PO_4 and distilled water to 1 liter, pH 7.1) to an initial absorbance (A_0) of 0.95–1.05 as measured in a Uvikon (Kontron, Zurich, Switzerland) 710 spectrophotometer at 400 nm. Bacterial suspensions (1.6 ml) were added to semi-micro, 1 cm square, (4 ml) disposable polystyrene cuvettes (Rudolf Brand, Wertheim, F.R.G.) and hexadecane volumes were then added as indicated. Mixtures were then vortexed directly in the cuvettes on a Thermolyne Maxi Mix flat top mixer (Sybron, Dubuque, IA) for 3- (*A. calcoaceticus* HO1-N) or 5-s (*S. pyogenes* M-5 and *S. marcescens* 3255) intervals. Following each mixing period the phases were allowed to separate and the absorbance of the lower aqueous phase (A_t) was then read directly. For each hexadecane volume, quadruplicate cuvettes were employed. The rate of adhesion, or removal rate, denoted as R, was calculated according to log A_t/A_0 as previously described [3]. Linear regression was employed to plot the results.

4. RESULTS AND DISCUSSION

The effect of the hydrocarbon : water ratio on the adherence rate of *A. calcoaceticus* HO1-N, a hydrocarbon degrading bacterium with a high affinity for hexadecane [5], is shown in Fig. 1. The logarithm of the percentage of cells removed from the bulk aqueous phase was directly proportional to the duration of mixing, enabling a rate to be derived for each hexadecane volume employed [3]. By plotting the rate of adherence as a function of the hydrocarbon : water ratio, a linear dependency was found (Fig. 1). The slope of this graph, denoted the removal coefficient [3,4], was 72 min^{-1} for this strain.

The effect of the growth temperature on the adherence kinetics of *S. marcescens* 3255 is shown in Fig. 2. Cells grown at 30 °C yielded a removal coefficient of 48 min^{-1}, whereas 35 °C-grown cells

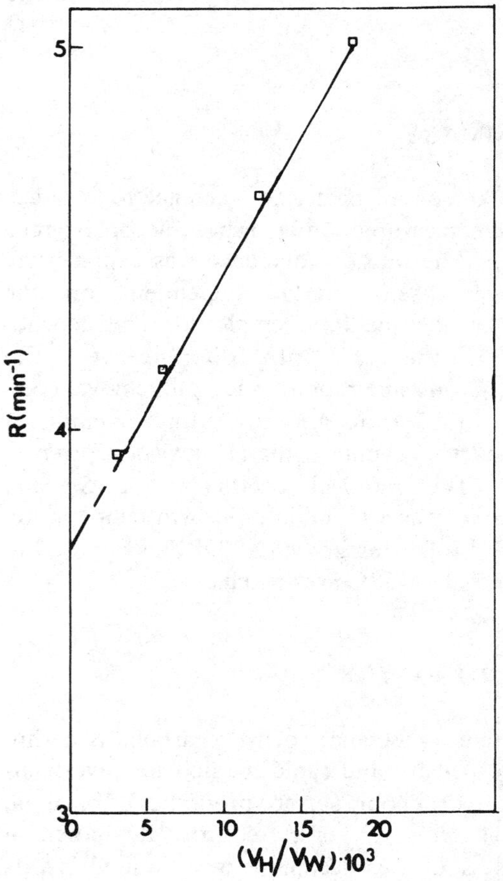

Fig. 1. Adherence of *A. calcoaceticus* HO1-N to hexadecane. The rate of adhesion, R, is plotted as a function of the hydrocarbon : water volume ratio.

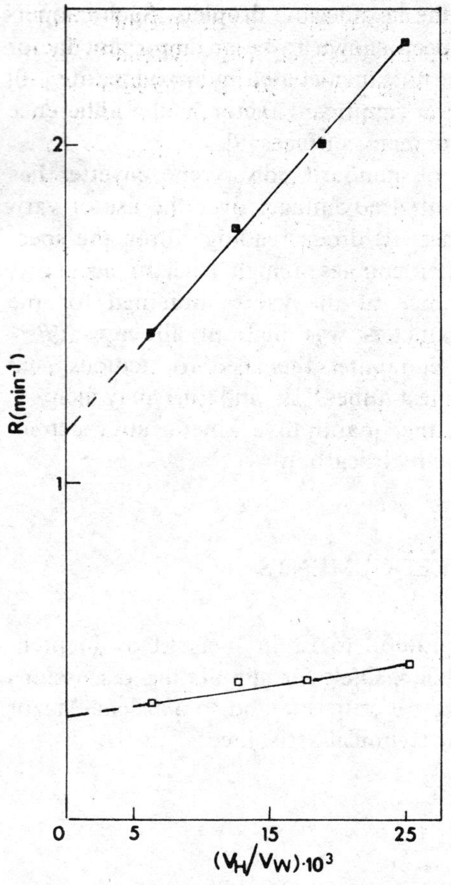

Fig. 2. Adherence of *S. marcescens* 3255 to hexadecane at two different growth temperatures. Cells were tested for adherence to hexadecane following growth at 30 (■) or 35 (□) °C. The rate of adherence, R, is plotted as a function of the hydrocarbon : water volume ratio.

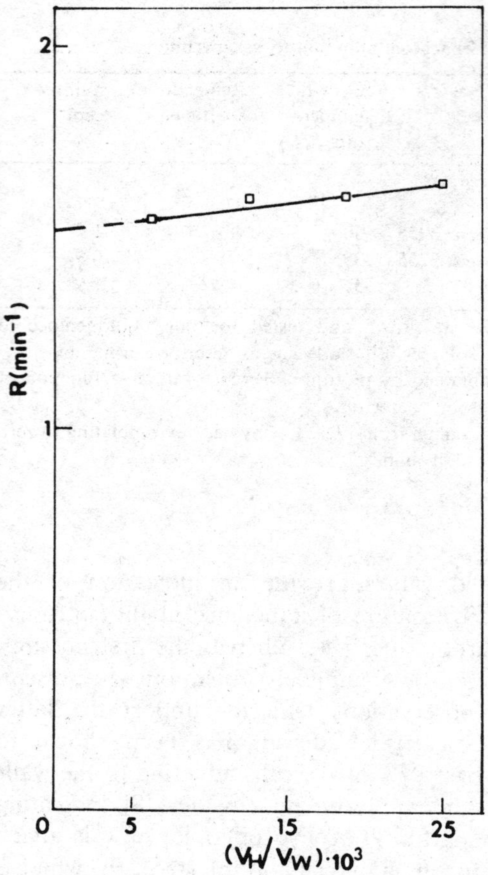

Fig. 3. Adhesion of *S. pyogenes* M-5 to hexadecane. The rate of adhesion, R, is plotted as a function of the hydrocarbon : water volume ratio.

were much less hydrophobic (removal coefficient of 5.8 min^{-1}). These results are in agreement with previous semi-quantitative results [7].

Somewhat surprising results were obtained with *S. pyogenes* M-5 (Fig. 3). Whereas rapid adhesion of the cells to hexadecane was observed, the rate of such adherence did not vary greatly with increasing hexadecane volumes, even at very low hydrocarbon : water ratios. The removal coefficient obtained was much less than that previously reported for the same strain [3]. However, this difference may be due to the finding that *S. pyogenes* hydrophobicity is extremely dependent on the physiological state of the cells, which may vary from laboratory to laboratory [6].

The quantitative data of the experiments are summarized in Table 1. Of considerable interest is the observation that back extrapolation of the dependence of rate of adherence on the hexadecane : water ratio gives significant removal coefficients at zero hexadecane volume. The magnitude of this back-extrapolated value varies from strain to strain and is not correlatable with the removal coefficients observed. For example, *S. pyogenes* has a relatively low removal coefficient but a high y-intercept value (Table 1). The non-zero values may be accounted for by two alternative explanations: (i) the non-zero values are due to adherence of the cells to the polystyrene cuvette rather than to the hydrocarbon phase; and (ii) the back-ex-

Table 1

Kinetics of bacterial adhesion to hexadecane

Strain [a]	Growth temperature (°C)	Removal coefficient [b]	Y-intercept [c]
A. calcoaceticus HO1-N	30	72	3.7
S. marcescens 3255	30	48	1.2
S. marcescens 3255	35	5.8	0.3
S. pyogenes M-5	37	4.7	1.5

[a] Strains were grown and tested for rate of adherence to various volumes of hexadecane as described in the text.

[b] Slope obtained by plotting adherence rate as a function of hydrocarbon : water ratio.

[c] Value obtained from Figs. 1–3 by back extrapolating to zero hexadecane volume.

trapolated values provide an indication of the cooperative nature of adherence at the aqueous : hydrocarbon interface. Whereas the first possibility cannot be completely ruled out at present, several observations tend to support the latter hypothesis: (i) hexadecane has been shown to desorb over 97% of the cells adhering to the walls of the same polystyrene cuvettes [8] indicating that the affinity of hydrophobic cells is much greater for the liquid : liquid interface; (ii) whereas extensive adherence to glass, or rather to the water film on the glass surface, is sometimes observed when the adherence assay is performed in glass tubes, we did not observe adherence to the polystyrene surface in the presence of hexadecane; (iii) the two strains which gave high back extrapolation values (i.e., *S. pyogenes* M-5 and *A. calcoaceticus* HO1-N) sometimes exhibit aggregation (unpublished observation), indicating high cell–cell affinity; (iv) adherence at the hydrocarbon : water interface often occurs in patches, (E. Rosenberg and M. Rosenberg, unpublished observations) suggested that cell : cell interactions do occur at the interface [9]. Based on these observations, we suggest that the back-extrapolated values may constitute an important parameter, expressing the degree of cell : cell cooperativity involved in their binding to the hexadecane droplets. Such cooperativity has been shown to be an important factor in adherence to saliva-coated hydroxylapatite [10] and may be a significant factor in the adherence of bacteria to many surfaces [9].

The use of standard polystyrene cuvettes has several potential advantages over the use of various test tubes: (i) direct reading within the spectrophotometer enables a high level of accuracy; the significance of the values obtained for the adherence kinetics was high in all cases ($P < 0.005$); (ii) eliminates the need for tedious acid washing of test tubes [1]; and (iii) may help to standardize the quantitative kinetic approach to adherence to hydrocarbon.

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REFERENCES

[1] Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) FEMS Microbiol. Lett. 9, 29–33.

[2] Rosenberg, M. (1984) FEMS Microbiol. Lett. 22, 289–295.

[3] Lichtenberg, D., Rosenberg, M., Sharfman, N. and Ofek, I. (1985) J. Microbiol. Methods 4, 141–146.

[4] Kawabata, N., Hayahsi, T. and Matsumoto, T. (1983) Appl. Environ. Microbiol. 46, 203–210.

[5] Kennedy, R.S., Finnerty, R.W., Sudarsanan, K. and Young, R.A. (1975) Arch. Microbiol. 102, 75–83.

[6] Ofek, I., Whitnack, E. and Beachey, E.H. (1983) J. Bacteriol. 154, 139–145.

[7] Rosenberg, M., Blumberger, Y., Judes, H., Bar-Ness, R., Rubinstein, E. and Mazor, Y. (1986) Infect. Immun. 51, 932–935.

[8] Rosenberg, M., Judes, H. and Weiss, E. (1983) J. Microbiol. Methods 1, 239–244.

[9] Rosenberg, M. and Kjelleberg, S. (1986) Adv. Microbiol. Ecol. 9, 353–393.

[10] Nesbitt, W.E., Doyle, R.J., Taylor, K.G., Staat, R.H. and Arnold, R. (1982) Infect. Immun. 35, 157–165.

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Concentration of larvicidal *Bacillus* spores at the water surface by adherence to oil droplets

Mel Rosenberg

School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978 (Israel)

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Summary

Adherence of two larvicidal spores, *Bacillus sphaericus* 2297 and *Bacillus thuringiensis israelensis,* to hexadecane and corn oil was determined. Adherence was greatly promoted in the presence of 2 M ammonium sulphate, reaching levels of up to 89%. The data suggest that larvicidal spores may be concentrated at their operational niche (the water surface) by flotation, adhering to the surface of oil droplets. The data suggest that adherence of the spores to oil droplets may enable their concentration at the water surface, where they can effectively kill mosquito larvae.

Key words: *Adherence* – Bacillus – *Larvicidal* – *Oil* – *Spores* – *Surface*

Introduction

Larvicidal bacillus spores are envisioned as an effective biological control agent in fighting mosquito growth and proliferation [1–3]. However, since the growth of the larvae is concentrated at the water surface, general application of the spores or toxins to the bulk aqueous phase is costly and ineffective. Since certain *Bacillus* spores are hydrophobic [4–7] and exhibit pronounced ability to adhere to oil : water interfaces [5, 6], it is conceivable that adherence to oil droplets may be a method of overcoming this problem.

Methods and Materials

To test this hypothesis, two strains of larvicidal *Bacillus* spores were tested for their ability to adhere to hexadecane and corn oil. Adherence to hexadecane and corn oil was carried out essentially as previously described [8, 9]. Cells of *Bacillus sphaericus* 2297 [1] and *Bacillus thuringiensis israelensis* [2] No. 1884 were grown on MBS sporulation media as described by Kalfon et al. [1] and washed in distilled water. For adherence, cells were suspended in phosphate buffer (11.1 g $KH_2PO_4 \cdot 3H_2O$,

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3.63 g KH_2PO_4), distilled water to 1 l, pH 7.1, in the absence or presence of 2 M ammonium sulphate, as indicated. To 1.2 ml of aqueous spore suspension, 0.4 ml of oil were added and the mixtures vortexed in standard acid-washed test tubes [8] for 2 min. Following phase separation, the absorbance of the lower aqueous phase was measured at 400 nm in a Uvikon 710 (Kontron, Zurich, Switzerland) spectrophotometer, and compared with that of the suspensions prior to mixing (initial absorbance of both strains was ca. 1.4 in the absence of ammonium sulphate, and ca. 1.2 in its presence). Results were expressed as the percent decrease in absorbance of the lower aqueous phase following the mixing procedure.

Results and Discussion

Typical results are shown in Table 1. Whereas adherence of both strains to either oil in phosphate buffer alone was low, very high levels of adherence of the two spore types to both oils were obtained when the assay was carried out in the presence of ammonium sulphate. Ammonium sulphate has been previously shown to enhance bacterial partitioning at the water:oil interface [9]. When the lower aqueous phase was replaced with water following the adherence assay, significant desorption of bound cells was not observed.

These results suggest that larvicidal *Bacillus* spores can be made to float on the water surface, if they are previously bound to oil droplets, preferably in the presence of a salting-out agent, such as ammonium sulphate. Moreover, such a non-covalent form of binding may result in a desirable slow release of bound spores at the feeding surface. The data show that the carrier may either be a liquid hydrocarbon, such as hexadecane, or a more biodegradable vegetable oil. The observed partitioning of larvicidal spores at the oil:water interface in the presence of ammonium sulphate may also provide a convenient means of separating endospores of larvicidal strains from vegetative cells and cell products (B. Sneh, personal communication).

TABLE 1

ADHERENCE OF LARVICIDAL *BACILLUS* SPORES TO OILS[a]

Strain	2 M Ammonium sulphate	Percent adherence to	
		n-Hexadecane	Corn oil
B. sphaericus 2297	Absent	14	40
	Present	88	90
B. thuringiensis israelensis	Absent	40	47
	Present	85	91

[a] Cells were grown, harvested, washed and suspended as described in the text. Adherence to 0.4 ml hexadecane (Fluka) or corn oil (Sigma) was performed in the absence or presence of 2 M ammonium sulphate. Results are expressed as the percent decrease in turbidity following the mixing procedure.

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References

1 Kalfon, A., Larget-Thiery, I., Charles, J.-F. and de Barjac, H. (1983) Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. Eur. J. Appl. Microbiol. Biotechnol. 18, 168 – 173.

2 Ohana, B., Margalit, J. and Barak, Z. (1985) A method for identifying colonies of bacteria possessing mosquito larvicidal effects. Appl. Microbiol. Biotechnol. 21, 250 – 251.

3 Lahkim-Tsror, L., Pascar-Gluzman, C., Margalit, J. and Barak, Z. (1983) Larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis*, serovar H14 in *Aedes aegypti*: histopathological studies. J. Invertebr. Pathol. 41, 104 – 116.

4 Rosenberg, M. (1984) Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22, 289 – 295.

5 Rosenberg, E., Brown, D.R. and Demain, A.L. (1985) The influence of gramicidin S on hydrophobicity of germinating *Bacillus brevis* spores. Arch. Microbiol. 142, 51 – 54.

6 Doyle, R.J., Nejdat-Halem, F. and Singh, J.S. (1984) Hydrophobic characteristics of *Bacillus* spores. Curr. Microbiol. 10, 329 – 332.

7 Boyles, W.A. and Lincoln, R.E. (1958) Separation and concentration of bacterial spores and vegetative cells by foam flotation. Appl. Microbiol. 6, 327 – 334.

8 Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Bacterial adherence to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29 – 33.

9 Rosenberg, M. (1984) Ammonium sulphate enhances adherence of *Escherichia coli* J-5 to hydrocarbon and polystyrene. FEMS Microbiol. Lett. 25, 41 – 45.

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The Borderland Between Caries And Periodontal Disease III

Edited by

T. Lehner

Department of Immunology,
Guy's Hospital Medical and Dental Schools, London, U.K.

and

G. Cimasoni

Section de Médecine Dentaire,
Université de Genève, Genève, Suisse.

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A novel microbial replica technique for localization of potential cariogenic growth on occlusal tooth surfaces

**M. ROSENBERG[1,2], E. WEISS[1], R. BAR-NESS[1]
D. FEDER[1] and I. ELI[1]**

INTRODUCTION

It is generally accepted that tooth morphology is an important factor in the development of dental caries. This is particularly true of occlusal tooth surfaces which can harbour microbial populations within deep pits and fissures which are not removable using standard oral hygiene techniques. If left untreated, such retentive occulsal sites often develop into carious lesions (Buonocore, 1972). Studies on actual (Loesche and Straffon, 1979; Loesche *et al.*, 1975; Meiers, Wirthlin and Shklair, 1982; Meiers and Schachtele, 1984; Theilade *et al.*, 1978) and artificial (Löe, Karring and Theilade, 1973; Theilade, Larson and Karring, 1973) fissures have found specific streptococci and lactobacilli to be predominant among the cultivable microbiota. For example, Theilade, Larson and Karring (1973) found that *Streptococcus sanguis* was present in high numbers in all the artificial fissures tested; the presence of *S. mutans* and lactobacilli appeared to increase with time. Loesche *et al.* (1975) sampled occlusal carious and non-carious fissures for *S. mutans* and *S. sanguis*; 71% of the carious fissures yielded high levels of *S. mutans*, whereas 70% of the caries-free fissures harboured no detectable *S. mutans*. However, 30% of the carious teeth did not yield *S. mutans*. Similar results were found in a

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[1] School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978 Israel.

[2] Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978 Israel.

study of interproximal lesions, in which *S. mutans* was not cultured in samples obtained from 28% of the carious lesions (Hoerman *et al.*, 1972). Such studies suggest that microbial examination of tooth surfaces can provide an indication of caries development, especially when several species are sampled.

At present, the main clinical technique for diagnosing occlusal caries is a subjective, manual test using a sharp explorer and dental mirror. In addition to its inaccuracy, this probing method has been criticized as exacerbating tooth destruction (Ekstrand and Thylstrup, 1985; van der Laan-van Oorp, Exterkate and Ten Cate, 1985). Thus, there appears a need for supplementary tests.

In the present report, we describe a novel approach (Rosenberg, Weiss and Eli, patent pending) to microbial sampling of occlusal tooth surfaces, based on an imprint (replica) technique. Whereas replica techniques have been previously employed, e.g. for candidal counts (Beavers *et al.*, 1985), they generally require gelling agents to fix the microbial growth. Such techniques are cumbersome and unsuitable for clinical practice. The method which we propose enables outgrowth of the imprint replica in liquid medium. Initial tests have shown that this approach (i) enables clear visualization of discrete growth loci, (ii) is amenable to clinical use, and (iii) may provide information on microbial retention sites which can be used to detect and possibly predict caries development.

MATERIALS AND METHODS

Development of technique

At the outset of this study, various liquid agar-containing media (Snyder agar, Rogosa agar and Mitis-Salivarius agar), cooled to 45° C, were poured onto freshly extracted teeth and allowed to gel. Following incubation for 48 h. at 37° C, areas of growth were clearly observed using Mitis-Salivarius agar. We then attempted various solid surfaces as potential replica matrices, including paper, silicone, alginate, latex and chewing gum base. Agar-containing media were then poured onto the replicas, as described above. Following 18 h. incubation, growth could be observed at various loci on several of these imprint materials. However, the process was difficult and cumbersome to perform. Often the medium gelled before complete contact with the replica surface was attained. Sometimes gas bubbles were trapped in the gel, making it hard to observe the bacterial growth below. In order to solve this problem, we attempted to incubate the replicas in liquid, rather than solid medium. In order to promote bacterial growth at the solid: liquid interface, rather than in the bulk liquid phase, we incorporated the main carbon and energy source, sucrose, within the replica matrix and removed it from the liquid medium composition. Following incubation in sucrose-free, chromogenic liquid media, discrete growth loci could be clearly observed, with little or no concomitant growth in the bulk liquid phase. Matrix and liquid medium compositions which gave satisfactory results as to the clarity with which growth could be discerned, are shown in Table I.

Initial clinical testing

Matrix and liquid medium compositions used were described in Table I. Volunteers (age 14-15) were requested to bite onto the matrix material at maximal occlusion. This procedure was performed in duplicate. The replicas were washed briefly in tap water, and incubated in petri dishes containing about 15 ml of the liquid medium, for 20 h. at 37° C. Following incubation, matrices were examined for microbial growth on the permanent first and second molar imprints, and scored as follows: 1 — no observable growth; 2 — weak growth; 3 — extensive microbial growth.

Each volunteer was clinically examined by a qualified dentist for existence of carious loci in the first and second permanent molars. Examination was carried out using a plain mouth mirror and a sharp, sickle-shaped explorer. Each molar was scored as follows: 1 — sound tooth; 2 — deep (sticky) fissure; 3 — definite caries.

RESULTS

Some examples of microbial growth on replicas are presented in Figure 1. Microbial growth appeared as discrete blue-black loci on the light back-

TABLE I

Major components of Solid Matrix and Liquid Outgrowth Medium

Liquid Medium	Solid Matrix
Tryptose	Commercial gum base
Proteose Peptone	Sucrose
Trypan Blue	
Gentian Violet	
Tellurite	
Distilled Water	

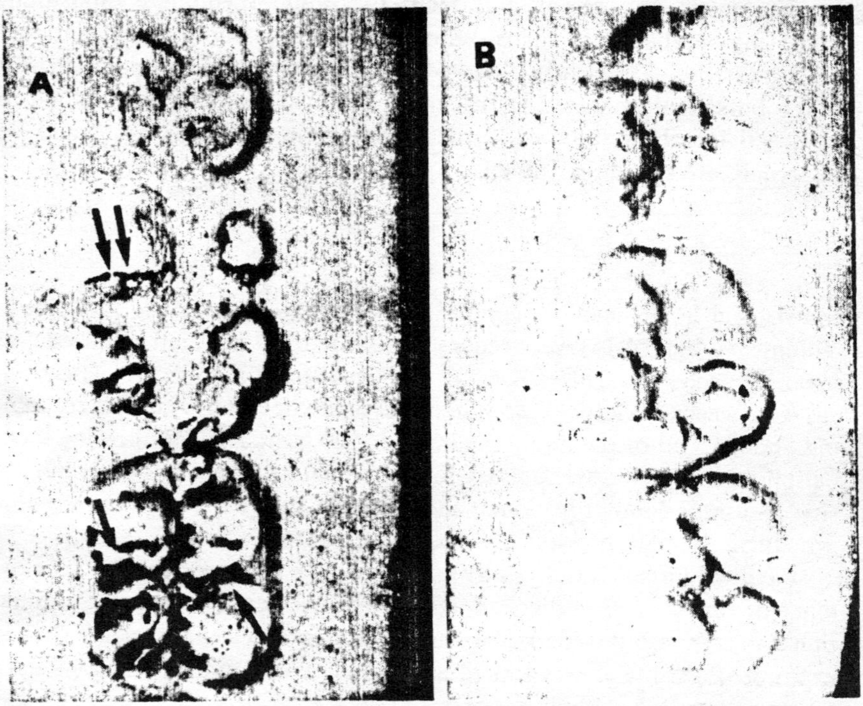

Fig. 1. Imprint replicas following incubation. Replicas were processed as described in Materials and Methods. A: Replica containing growth loci on sites corresponding to occlusal fissures (single arrows) and uneven restoration margins (double arrow); B: Replica free of microbial growth.

ground of the replica surface. Growth could be detected along certain deep occlusal fissures or at uneven margins of the dental restorations.

Preliminary results of a clinical study of the permanent molars of 114 Israeli high school students are shown in Figure 2. A trend was observed in microbial growth on the replica, as a function of the clinical status of the tooth. Thus, the ratio of extensive compared with no growth on the replica was 2.0:1.0 for teeth diagnosed as healthy (clinical status 1), 4.6:1.0 for teeth diagnosed as possessing deep (sticky) fissures (clinical status 2), and 8.0:1.0 for teeth diagnosed as carious (clinical status 3). However, among the teeth clearly diagnosed as carious (clinical status 3), 7.8% gave no observable growth on the corresponding replica. This could be due to: (i) improper replica taking; (ii) lack of replication of microorganisms at the carious loci onto the matrix; (iii) lack of growth of these microorganisms using the particular liquid medium: matrix combination; (iv) improper clinical diagnosis.

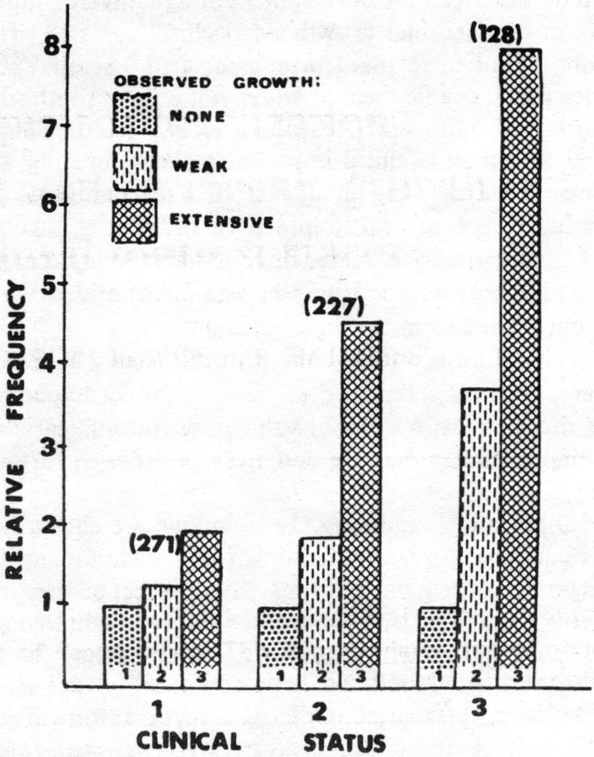

Fig. 2. Initial clinical study of permanent molars of Israeli high school students. Teeth were scored for clinical status and growth on replica as dscribed in Materials and Methods. Results are presented as relative frequency of each growth category as a function of clinical status; numbers in parenthesis indicate total number of teeth in each clinical status group.

DISCUSSION

In recent years increasing interest has focused on the possibility that dental caries may be identifiable at the early stages of development. Among the tests proposed for the diagnosis and prediction of caries are various microbiological assays measuring growth of caries-associated bacteria and/or their metabolic products. Examples which have found some clinical application are tests for *Streptococcus mutans* and lactobacilli, and acid production by aciduric and acidogenic oral microbiota (Klock and Krasse, 1979; Newbrun, 1983). However, whereas such tests indicate a general predisposition towards caries development, they do not attempt to pinpoint the carious loci.

We have described the development of a relatively simple technique for localization of microbial growth on occlusal surfaces. The technique is based on the premise that caries-associated bacteria possess adhesive properties which enable them to attach not only to tooth surfaces, but to a wide variety of substrata (Rosenberg *et al.*, 1983). This approach was employed to obtain occlusal imprints (replicas) bearing adherent microorganisms. Replicas were incubated in a chromogenic liquid medium selective for growth of oral streptococci. In order to encourage growth at the solid: liquid interface, rather than in the bulk liquid medium, the main carbon and energy source (sucrose) was incorporated within the replica matrix, but absent from the liquid medium.

The general observation that growth occurs at sites of microbial retention and caries potential (i.e., fissures and/or imperfect restorations) suggests that this test may be of value in diagnosing caries-prone occlusal sites. Longitudinal studies are underway in order to further examine this possibility.

Several questions regarding the technique are currently under investigation, including: (i) determination of the predominant microorganisms following outgrowth at various sites; (ii) the effect of altering the selectivity of the growth medium; and (iii) the possibility of employing the technique to assess microbial growth following occlusal sealing.

SUMMARY

In order to locate carious and pre-carious growth on the tooth surface, a novel replica system has been developed. The test is based on the following principles: (i) obtaining an impression replica of the occlusal tooth surfaces onto a malleable solid matrix. At this stage, adherent cariogenic microorganisms are replicated onto the impression surface. (ii) Outgrowth of the replicated microorganisms in a liquid medium selective for *Streptococcus mutans* and other cariogenic streptococci; this contains trypan blue, Gentian violet and potassium tellurite. In order to confine microbial growth to the solid: liquid interface, the solid matrix is impregnated with a necessary growth substrate which is absent from the liquid medium (e.g. sucrose as major carbon and energy source). (iii) Identification of carious and potentially carious sites, according to the corresponding loci of bacterial growth on the impression surface.

The test is carried out in four steps: (i) impression taking; (ii) washing the replica surface to remove non-bound bacteria (e.g. from saliva); (iii) overnight incubation in the liquid medium and (iv) observation of growth

loci. The simplicity of the test enables it to be carried out by paramedical personnel. Preliminary experiments in this laboratory have shown that microbial growth on the replica surface is confined to either sites which are carious, or to sites of potential caries development, such as deep pits, fissures and defective restorations. Initial results suggest that this technique is a simple, inexpensive way in locating caries and predicting loci of carious development.

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REFERENCES

Beavers, F.P.P., Wilson, M., Ivanyi, L. and Hobkirk, J.A. (1985). *J. Dent. Res.* **63**, 519.

Buonocore, M.G. (1972). *Dental Clin. N. Am.* **16**, 693.

Ekstrand, K. and Thylstrup, A. (1985). ORCA XXXII Congress Abstract Book, pp. v-10.

Hoerman, K.C., Keene, H.J., Shklair, I.L. and Burmeister, J.A. (1972). *JADA* **85**, 1349.

Klock, B. and Krasse, B. (1979). *Scand. J. Dent. Res.* **87**, 129.

Löe, H., Karring and Theilade, E. (1973). *Caries Res.* **7**, 210.

Loesche, W.J. and Straffon, L.H. (1979). *Infect. Immun.* **26**, 498.

Loesche, W.J., Rowan, J., Straffon, L.H. and Loss, P.J. (1975). *Infect. Immun.* **11**, 1252.

Meiers, J.C., Wirthlin, M.R. and Shklair, I.L. (1982). *J. Dent. Res.* **61**, 460.

Meiers, J.C. and Schachtele, C.F. (1984). *J. Dent. Res.* **63**, 47.

Newbrun, E. (1983). Cariology, Williams & Wilkins, Baltimore, pp. 256-273.

Rosenberg, M., Rosenberg, E., Judes, H. and Weiss, E. (1983). *FEMS Microbiol. Lett.* **20**, 1.

Rosenberg, M., Weiss, E. and Eli, I., U.S. patent pending.

Theilade, E., Larson, R.H. and Karring, Th. (1973). *Caries Res.* **7**, 130.

Theilade, E., Fejerskov, O., Karring, Th. and Theilade, J. (1978). *Caries Res.* **12**, 313.

van der Laan-van Oorp, C.S.F., Exterkate, R.A.M. and Ten Cate, J.M. (1985). ORCA XXXII Congress Abstract Book, pp. v-9.

הליטוזיס — מדידה וטיפול

מל רוזנברג־נבו*, יעקב גבאי**

בית הספר לרפואת שיניים ע״ש מוריס וגבריאלה גולדשלגר והפקולטה לרפואה ע״ש סאקלר, אוניברסיטת תל־אביב

ריח־פה רע (הליטוזיס) מצטייר כתופעה אוניברסלית, או כמעט אוניברסלית, אצל מבוגרים ברחבי העולם.

בארה״ב בלבד מוציאים מעל 500 מיליון דולר לשנה על שוטפי פה, תרסיסים ומוצרים נלווים (שלמרבית האירוניה, השפעתם מתבטאת במיסוך זמני של הריח ללא השפעה מהותית). למרות היקפה של הבעיה והתפתחות מודעות בריאות הפה בעולם המערבי, עדיין לא נעשה נסיון לטפל בגורמים האחראים לריח הרע במרפאות השיניים. מאמר זה דן בבעיה ומציע דרך למדידה, טיפול ומעקב אחרי ריח זה.

מהם הגורמים לריח הרע? במרבית המקרים (2,1) התשובה היא חיידקי הפה. הריח יכול לנבוע מהצטברות חיידקים ברוק העומד זמן ממושך ובאבן השן, ריקבון מתקדם, דלקת חניכיים מוקדמת ומחלות פריודונטליות, בסמוך לשיניים תותבות, במירווחים בין השיניים, על הלשון והריקמה הרירית של חלל הפה, בכיבים וכו׳ (1־4).

חלקיקי מזון לכודים, תאי אפיתל ופסולת אחרת מחמירים את עוצמת הריח, כנראה כתוצאה מפרוק הנגרם על־ידי מיקרואורגניזמים הנמצאים בסביבה (2). המטבוליזם של חיידקי הפה יכול ליצור גזים בעלי ריח רע (2, 5־7), רובם תרכובות גופרית כמו מימן גופרי, מתיל ואתיל מרקפטאן ודי־מתיל גופרי (2).

במרבית המקרים ניתן להקטין את בעיית הצטברות החיידקים הנ״ל בחלל הפה בעזרת טיפולי־פה סטנדרטיים בשילוב עם שמירה על בריאות הפה. הברשה או גרוד הלשון (2,1) והריקמה הרירית עשויים להקטין את מימדי הריח, כך ש־85%־90 מהמקרים של ריח־פה הנובעים מבעיות בחלל הפה (2,1), לפחות תיאורטית, ניתנים לטפול.

ההתעניינות הראשונית שלנו בהליטוזיס החלה לפני מספר שנים במהלך פיתוחו של שוטף־פה חדש על בסיס שמן־מים אשר קושר ומרחיק חיידקים בחלל הפה וסופג ריחות רעים מהרוק (8). במהלך שיחות והתכתבויות עם חולים ורופאי שיניים הסתבר לנו שאנשים הסובלים מריח רע בפיהם מטופלים רק לעיתים נדירות על־ידי רופאי השיניים שלהם אשר בדרך־כלל מפנים אותם למומחים אחרים כמו גסטרואנטרלוגים אשר מצידם מפנים אותם חזרה לרופאי השניים. אחד ההסברים לתופעה זו הינו שרופאי שיניים לא ששים לטפל בהליטוזיס כשלעצמו משום שאין ברשותם מדד כמותי להערכת התופעה, דבר שאינו מאפשר לקבוע במדוייק את מידת ההצלחה של הטיפול.

ניתן לבצע מדידות כמותיות של גזים מסויימים בהליטוזיס בעזרת גז כרומטוגרף (7,5,3,2), אך שיטה זו הינה יקרה ומורכבת מדי מכדי לשמש למדידות שיגרתיות. הועלתה אפשרות לעשות שימוש במכשיר ניטור תעשייתי זול למדידת הריכוז הכללי של תרכובות גופרית נדיפות.

בתגובה לראיון ברדיו בשנה שעברה, פנו אלינו מעל ל־180 מבוגרים מאיזור ת״א אשר התלוננו על הליטוזיס. הופתענו ממספר הפונים במיוחד לאור העובדה שהצענו רק לנסות ולמדוד את רמת ההליטוזיס בפיהם ללא התייחסות לתוכנית טיפול כלשהיא. במהלך החודשים שלאחר מכן ביצענו עבודה בה בחנו את יכולתו של חיישן תעשייתי לתרכובות גופרית (תמונה 1 ו־2) למדוד הליטוזיס בקבוצת מתנדבים בהשוואה לקבוצה אחרת של סטודנטים לרפואת שיניים ואנשי סגל בית ספרנו אשר נבחרו באקראי. דו״ח ראשוני המתייחס לעבודה זו הוצג לאחרונה (9). דו״ח מפורט יותר נמצא בשלבי עיבוד. הנתונים הכמותיים שהושגו באמצעות חיישן הגופרית הושוו עם תוצאות שהתקבלו מצוות מריחים (בדיקה אור־ גנולפטית) שכלל שבעה מתנדבים שכל אחד מהם דרג את מידת הריח של כל נבדק לפי סקלה שבה 0 מייצג חוסר תחושה של ריח רע עד דרגה 5 שהתאימה לריח בלתי־נסבל. מבחן הריח מוצג בתמונה 3. ככלל, נמצא מתאם חיובי בין שתי השיטות, דבר המרמז על כך שחיישן תרכובות הגופרית שעבר שינויים מסויימים עשוי אכן לשמש כאמצעי מדידה לריח רע.

נתונים אלה והנסיון הראשוני שלנו מאפשרים לגבש מודל לטיפול בחולים הסובלים מריח רע. בשלב הראשון נבדק הפונה לטיפול בעזרת החיישן במקביל למבחן איכותי על־ידי מיבדק אורגנולפטי (10). היות וקיימת סבירות נמוכה שהשימוש בחיישנים מסוג זה יהיה נפוץ בעתיד הקרוב, ניתן להפנות את החולה למרפאה העוסקת באיבחון בעיות הפה.

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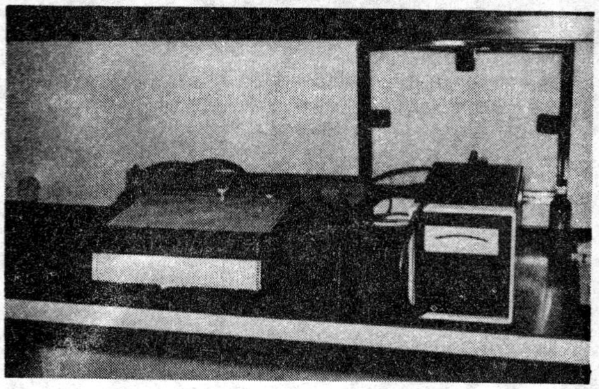
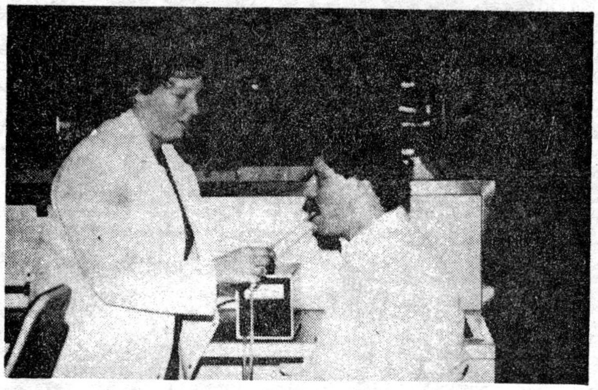
* המעבדה למיקרוביולוגיה אורלית והמחלקה למיקרוביולוגיה הומנית

** המכון לחקר בריאות הסביבה, משרד הבריאות



תמונה 1: מכשיר תעשייתי למדידת סולפידים (תרכובות גופרית) המחובר לעט רושם.



תמונה 2: מדידה כמותית של הליטוזיס. צינור פלסטיק המחובר למכשיר (במרכז התמונה) מוכנס לחלל הפה של הנבדק (מימין) לצורך בדיקה כמותית.

תמונה 3: מדידה אורגנולפטית: מדידת עוצמת הריח בחלל הפה של הנבדק (מימין) המבוצע על־ידי מתנדב מצוות המריחים.

הפנייה מסוג זה עשויה להיות בעלת יתרונות נוספים. לדוגמא, חולים מצחצחים לעיתים את השיניים או משתמשים בשוטף־פה או בתרסיס לפני הגיעם לטיפול שיניים, דבר המקשה על רופא השיניים להעריך את רמת הריח. יתר על כן, ניתן לבקש מחולים להיבדק כאשר רמת הריח הינה גבוהה (בשעות הבוקר המוקדמות או שעות אחה"צ המאוחרות ולא בזמן הביקור אצל הרופא).

לחולים שהתגלתה אצלם רמה משמעותית של הליטוזיס בעקבות שני המיבדקים הנ"ל, יומלץ לעבור טיפול דנטלי ופריודונטלי מלא שיכלול גם חינוך להיגיינת־פה נאותה. נמצא שצחצוח שיניים ושימוש בחוט דנטלי הינם יעילים מאוד בהפחתה לזמן קצר של ריחות הנובעים מתרכובות גופרית נדיפות (2.1). גרוד יומי של הלשון (1) והברשה של ריקמת הפה הרירית עשויים להפחית עוד יותר את הריח. במהלך הטיפול יש להפנות את המטופל מדי פעם לבדיקות־ביניים. ניתן להעריך את מידת היעילות של כל שלב בטיפול. באופן דומה, צריכות ביקורות שנערכות לאחר הטיפול לכלול גם מדידות של הליטוזיס. יתכן שאצל מספר חולים ימשיכו להתקבל רמות הליטוזיס גבוהות למרות שעברו טיפול אורלי והקפידו על היגיינת הפה. דבר זה עשוי להצביע על כך שמקור הריח הרע אינו בחלל הפה ויש לחפש מקורות אחרים (1). יש לזכור שבדרך־כלל אנשים עם ריח־פה אינם מודעים לבעיה אלא אם כן הדבר הובא לתשומת לבם על־ידי חברים או קרובים (1). כך, חולים שיש להם ריח־פה אך מעולם לא העירו להם על כך אינם סובלים מהבעיה. לחולים שהבדיקות המעבדתיות שלהם מראות שאין להם הליטוזיס ולמרות זאת מתלוננים על ריח רע מהפה יש לערוך מדידות נוספות בשעות שונות של היום. אין ספק שבעקבות תוצאות שליליות חוזרות ונשנות ישוכנעו חלק מהחולים שאכן הבעיה אצלם אינה משמעותית. לאחרים שימשיכו לעמוד על כך שהם סובלים מריח־פה יש אולי בעיה פסיכולוגית (1) וניתן להפנותם לייעוץ מתאים בנושא.

רשימת הספרות מופיעה בחלק האנגלי.

Halitosis — A Call for Affirmative Action

Mel Rosenberg, Ph.D., Jacob Gabbay, Ph.D.***

THE MAURICE AND GABRIELA GOLDSCHLEGER SCHOOL OF DENTAL MEDICINE, AND SACKLER FACULTY OF MEDICINE, TEL-AVIV UNIVERSITY

Bad breath (halitosis) appears to be a universal, or almost universal, problem among adults throughout the world. In the U.S.A. alone, over $500 million are spent annually on mouthwashes, sprays, and related over-the-counter products (ironically, many of these have little actual effect, other than masking the smell for a short period!). Despite the magnitude of this problem, and the growing oral-hygiene awareness in the Western hemisphere, bad breath *per se* is not commonly treated in the dental clinic. The present paper addresses this issue, and suggests a regimen for measurement, treatment and follow-up of oral halitosis.

What are the factors responsible for halitosis? In the vast majority of cases (1,2), the answer is oral bacteria. Halitosis may stem from bacterial accumulations in stagnant saliva, dental plaque, advanced caries, early gingivitis and periodontal diseases, in the vicinity of prosthetic appliances and interdental spaces, on the tongue and buccal mucosa, in absesses, and so on (1-4); trapped food particles, epithelial cells and other debris can exacerbate halitosis, presumably due to their degradation by adjacent microorganisms (2). The metabolism of oral bacteria results in the production of putrid volatile gases (2,5-7). Among these, sulphur-containing molecules, such as hydrogen sulphide, methyl and ethyl mercaptan, and dimethyl sulphide, predominate (2).

In most cases, the abovementioned bacterial accumulations in the oral cavity can be alleviated by standard oral treatments, combined with regular oral hygiene. Brushing or scraping of the tongue (1,2) and buccal mucosa may also reduce halitosis. Thus, 85-90% of halitosis cases emanating from oral problems (1,2) are amenable, at least theoretically, to treatment. Our original interest in halitosis was initiated several years ago, during the development of a novel oil: water mouthwash which binds and removes oral bacteria and absorbs odours from putrefied saliva (8). During the course of conversations and correspondence with patients and dentists, we learned that people complaining of bad breath are seldom treated for this ailment by their dentists, and are often referred to other specialists, e.g. gastroenterologists, who, in turn, refer them back to dental practitioners. One possible explanation for this phenomenon is that dentists are reluctant to treat halitosis per se because they have no quantitative criteria with which to measure bad breath, and thus cannot accurately judge the success of their treatment regimen.

Whereas quantitative measurements of the individual gases in the oral air space can be made using gas chromatography (2,3,5,7), this procedure is too expensive and complicated to be used as a routine measurement. However, the possibility was raised that a relatively inexpensive industrial analyzer, which provides an overall concentration of volatile sulphides, might suffice for this purpose.

In response to a local radio interview with one of us last year, over 180 adults from the Tel-Aviv area, complaining of halitosis, contacted the laboratory. We were surprised by the number of referrals, especially since we offered only to attempt to **measure** their halitosis levels, and not to attempt any kind of actual treatment. During the following months, we performed a study, in which we tested the ability of an industrial sulphide sensor (Figs. 1,2) to measure halitosis in a group of these volunteers, as compared to

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* Laboratory of Oral Microbiology and Department of Human Microbiology.

** Research Institute for Environmental Health, Ministry of Health, Israel.

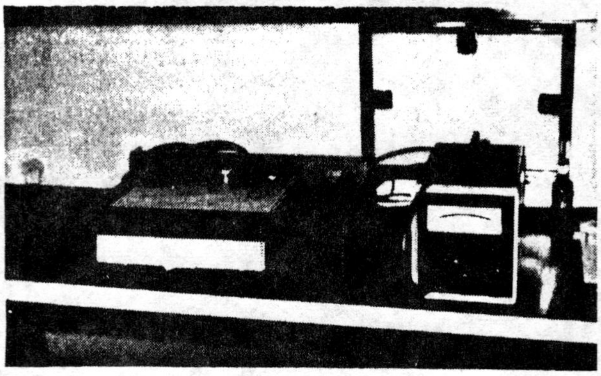

Fig. 1: Interscan sulphide analyzer (right) connected to pen recorder (left).

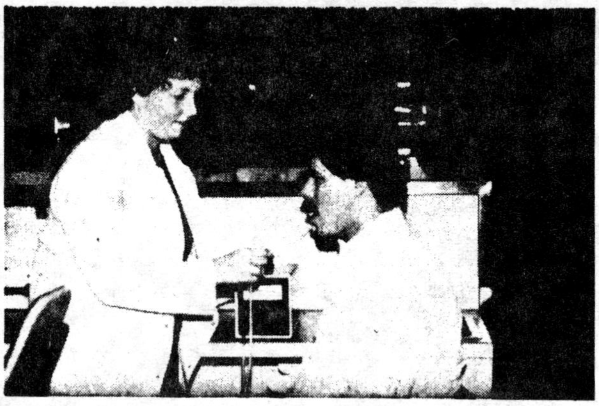

Fig. 2: Quantitative measurement of halitosis. Plastic tube, connected to analyzer (center), is inserted into open mouth of subject (right) for quantitative measurement.

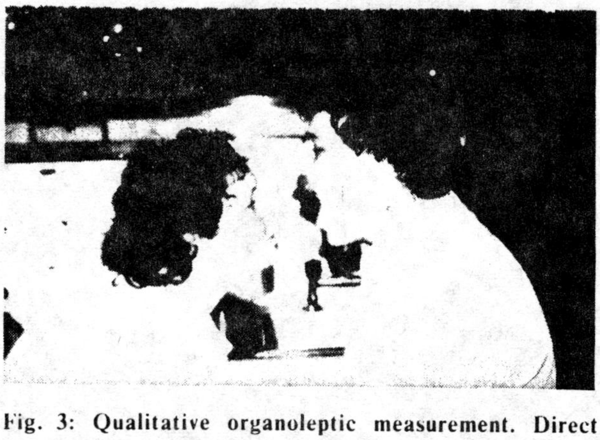

Fig. 3: Qualitative organoleptic measurement. Direct organoleptic measurement of the odor level of subject's oral air space (right) by member of panel (left).

a group of randomly selected dental students and staff of our School. A preliminary report of this pilot study was recently presented (9); a detailed account is in preparation. The quantitative data obtained by the sulphide sensor were compared with the results of an organoleptic panel which included seven volunteers, each grading the smell of each patient according to a qualitative scale from no observable odor — 0, to unbearably foul odor — 5. The organoleptic test is illustrated in Fig. 3. In general, positive correlations were found between the two methods, suggesting that a modified sulphide sensor can indeed measure bad breath.

These data, and our initial experience, suggest the following model for treatment of patients complaining of oral malodor. In the first instance, any patient suffering from halitosis is tested using a sulphide sensor, in conjunction with a qualitative organoleptic test (10). Since it is unlikely that suitable sulphide sensors will become widely available in the very near future, the patient may be referred to an oral diagnosis clinic which has expertise in this area. Referral to such a clinic may have additional advantages. For example, patients sometimes brush their teeth or use mouthwash or oral sprays immediately prior to dental appointments, making it difficult for the dentist to assess halitosis levels. Moreover, patients can be requested to take the test when halitosis levels are high (early morning, late afternoon), rather than during the dental appointment.

Patients who are found to have significant levels of halitosis, following both tests, should be encouraged to undergo complete dental and periodontal treatment, including education towards proper oral hygiene. Regular toothbrushing and flossing have been shown to be highly effective in short-term reduction of oral sulphur volatiles (1,2). Daily scraping of the tongue (1) and brushing of the oral mucosa may further reduce halitosis. At various times during the treatment, the patient should be referred for interim tests. In this manner, the effectiveness of each of the treatment phases can be appraised. Similarly, halitosis measurements should accompany subsequent check-ups. Some patients may continue to have high halitosis readings, even following through oral treatment and stringent oral hygiene; this might suggest that the bad breath does not originate in the oral cavity, and that alternative sources be sought (1). It should be kept in mind that people with bad breath are generally unaware of it, unless alerted by family or friends (1). Thus, patients who complain of halitosis, but have never been told that they do have bad breath, may not actually suffer from offensive oral malodor. Patients who do not have halitosis, according to the laboratory tests, yet insist that they have foul breath, should be subjected to additional measurements, e.g. at different times of day. Undoubtedly, following repeated negative results, some patients will be reassured that they do not suffer appreciably from halitosis. Others, who continue to insist that they have bad-breath, despite repeated results to the contrary, may harbour a psychological problem (11), and can be referred to appropriate counsel.

REFERENCES

1. Spouge, J.D.: Halitosis, a review of its causes and treatment, The Dental Practitioner and Dental Record, 14:307-317, 1964.
2. Tonzetich, J.: Production and origin of oral malodor: a review of mechanisms and methods of analysis, J. Periodontol. 48:13-20, 1977.
3. Kostelc, J.C., Preti, G., Zelson, P.R., Brauner, L. and Baehni, P.: Oral odors in early experimental gingivitis, J. Perio. Res. 19:303-312.
4. Dayan, D., Begleiter, A. and Buchner, A.: Halitosis and fetor oris, Quintessence International, 8:1-3, 1982.
5. Solis-Gaffar, M.C., Niles, H.P., Rainieri, W.C. and Kestenbaum, C.: Instrumental evaluation of mouth odor in a human clinical study, J. Dent. Res. 54:351-357, 1975.
6. Solis-Gaffar, M.C., Rustogi, K.N. and Gaffar, A.: Hydrogen sulfide production from gingival crevicular fluid, J. Periodontol. 51:603—606.
7. Kostelc, J.G., Zelson, P.R., Preti, G. and Tonzetich, J.: Quantitative differences in volatiles from healthy mouths and mouths with periodontitis.
8. Weiss, E., Rosenberg, M. and Judes, H.: Dental and oral preparation. U.S. patent 4,525,242.
9. Pshigorski, I., Gabbay, J., Eli, I., Brenner, S., Bar-Ness, R. and Rosenberg, M.: A novel approach to halitosis measurement. J. Dent. Res. (abs.; in press).
10. Pitts, G., Pianotti, R., Feary, T.W., McGuiness, J. and Masurat, T.: The in vivo effects of an antiseptic mouthwash on odor-producing microorganisms, J. Dent. Res. 60:1891—1896, 1981.
11. Beary, M.D. and Cobb, J.P.: Solitary psychosis — three cases of monosymptomatic delusion of alimentary stench treated with behavioural psychotherapy. Brit. J. Psychiat. 138:64—66.

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Clearance of *Serratia marcescens* from Blood in Mice: Role of Hydrophobic Versus Mannose-Sensitive Interactions

SHIMON RUMELT,[1] ZVI METZGER,[1] NAAM KARIV,[2] AND MEL ROSENBERG[1,3]*

The Maurice and Gabriela Goldschleger School of Dental Medicine,[1] Glasberg Tower for Medical Research,[2] and Department of Human Microbiology,[3] Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, 69978 Tel-Aviv, Israel

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In the present study, we examined the potential roles of cell surface hydrophobicity and mannose-sensitive (MS) interactions in blood clearance of *Serratia marcescens* in mice. Hydrophobic strain RZ, partially hydrophobic mutant 3162, and nonhydrophobic mutant 3164 were coinoculated into BALB/c male mice, and blood samples were plated out at different time intervals; colonies of the three strains were distinguished by their different morphologies. All three strains were cleared from the blood stream at similar rates, despite their large relative differences in cell surface hydrophobicity. Clearance from blood was subsequently studied by coinoculating two clinical isolates which differ in their abilities to adhere via MS interactions. MS$^+$ strain 1785 was cleared much more rapidly than MS$^-$ strain 3255; moreover, in the presence of D-mannose, clearance of strain 1785 was inhibited to a rate similar to that of MS$^-$ strain 3255. When D-glucose was substituted for D-mannose, inhibition was not observed. The results suggest that MS, rather than hydrophobic, interactions are primarily responsible for the rapid clearance of *S. marcescens* from blood observed.

Bacterial adhesion has been recognized as an initial step in many instances of host colonization (1). Many adhesion phenomena have been attributed to either hydrophobic interactions or lectinlike (e.g., mannose-sensitive) interactions (1, 2–5, 7–9, 11–15). *Serratia marcescens* is an interesting microorganism in this respect, since strains may independently exhibit either or both adhesion mechanisms (13).

Cell surface hydrophobicity has been cited as an important factor in mediating bacterial adhesion to a variety of surfaces of clinical importance, including tooth surfaces, plastic implants, epithelial cells, and phagocytes (2, 4, 8, 12, 13, 15; for a review, see reference 14). However, little attention has been paid to the possible effects of bacterial hydrophobicity on microbial survival after entry into the bloodstream. Ofek et al. (8) have postulated that *Streptococcus pyogenes* may alter its outer surface hydrophobicity, presenting a hydrophobic outer surface to promote initial adhesion but substituting a hydrophilic hyaluronic acid capsule after invasion to evade clearance mechanisms. The recent finding that clinical *S. marcescens* isolates exhibit pronounced hydrophobicity when grown at 30°C but not at 38°C led to the similar conjecture that modulation of cell surface hydrophobicity may serve as a clearance-avoiding mechanism in *S. marcescens* (13).

Recent studies with *Escherichia coli* (9) and *Salmonella typhimurium* (3, 5) have shown that clearance of these microorganisms from blood is primarily due to mannose-sensitive (MS) interactions. However, neither of these bacteria normally exhibits pronounced cell surface hydrophobicity (14).

In the present study, we examined the potential roles of cell surface hydrophobicity and MS interactions in mediating clearance of *S. marcescens* from blood in mice. The data suggest that MS interactions, not bacterial hydrophobicity, are mainly responsible for the rapid clearance from blood observed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. marcescens* RZ was originally obtained from R. Zack, Tel-Aviv University. Mutants 3162, exhibiting intermediate cell surface hydrophobicity, and 3164, almost completely devoid of cell surface hydrophobicity, were derived from strain RZ, as reported previously (12). Pigmented clinical isolate 1785 was obtained from the Rambam Hospital, Haifa, Israel; nonpigmented isolate 3255 was obtained from the Chaim Sheba Medical Center, Tel-Hashomer, Israel (13). Relevant properties of the strains used are presented in Table 1. The bacteria were maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) at 4°C and transferred every month. Starter cultures were prepared by inoculating bacteria to tubes containing 4 ml of BHI broth (Difco) which were incubated for 18 h with shaking at 30°C. Bacteria were then inoculated into flasks containing 50 ml of BHI broth with 0.25 ml of liquid culture and incubated for 18 h at 30°C in a G76 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 300 rpm. After growth, bacteria were harvested by centrifugation at 10,000 × *g*, washed twice, and suspended in a phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, distilled water to 1 liter [pH 7.2]) to an optical density of ca. 1.0 at 400 nm, as measured with a Kontron 710 spectrophotometer (Uvikon, Zurich, Switzerland). Suspensions were tested for cell surface hydrophobicity on the bases of (i) adhesion to 0.2 ml of hexadecane, as previously described (12, 13) but with PBS as the suspending buffer, and (ii) adhesion to untreated polystyrene (13); MS adhesion (6, 7) was determined by mannose-inhibitable aggregation of *Saccharomyces cerevisiae* cells as described previously (9, 13).

Survival of bacteria in fresh mouse blood in vitro. To 0.5 ml of fresh mouse blood was added 0.05 ml of a bacterial suspension containing ca. 10^6 bacteria per ml and heparin (20 U). The mixtures were tumbled at 37°C for 60 min. The suspensions were then diluted 1:10 in distilled water and subsequently in PBS and plated on BHI agar. Test tubes

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* Corresponding author.

TABLE 1. Properties of the *S. marcescens* strains used

Strain	Distinguishing colony morphology	Cell surface hydrophobicity[a]	MS adhesion[b]
RZ	Pigmented	High	+
3162	Nonpigmented	Intermediate	+
3164	Pigmented, translucent[c]	Low	+
1785	Pigmented	High	+
3255	Nonpigmented	High	−

[a] As determined by percent adhesion to 0.2 ml of hexadecane (13); high, >75%; intermediate, 25 to 75%; low, <25%.

[b] As determined by mannose-inhibitable agglutination of *S. cerevisiae* cells (6, 13).

[c] With respect to wild-type RZ colonies.

containing heparinized PBS instead of blood served as controls.

Clearance of bacteria from blood. Male BALB/c mice (age, 6 to 7 weeks; three mice in each group) were injected by the tail vein with 0.5 ml containing bacterial mixtures (ca. 10^8 CFU/ml total) and 50 U of heparin (to test the effects of sugars, the mixture was supplemented with 90 mg of D-mannose or D-glucose, as indicated; such concentrations were found to be below the lethal level). At various times after injection, 20-μl samples of blood were obtained from the retroorbital plexus with disposable micropipettes. The samples were diluted 1:10 into distilled water and subsequently in PBS and plated on BHI agar. Differentiation between the different strains was based on colony morphology and pigmentation (Table 1).

Statistical analysis. Statistical analysis was carried out with the paired *t* test.

RESULTS

The survival of the various bacterial strains in fresh mouse blood is shown in Table 2. In no instance was killing observed during a 60-min period.

Figure 1 represents a typical experiment showing the blood clearance of three isogenic strains that differ in their hydrophobic properties: wild-type strain RZ, which exhibits pronounced cell surface hydrophobicity; mutant 3162, which exhibits intermediate affinity for hydrophobic surfaces; and nonhydrophobic mutant 3164 (13). All three strains were cleared from the blood stream at similar rates, despite the extreme differences in their respective surface hydrophobicities.

Clearance from blood of two clinical isolates, 1785 and 3255, which have similar hydrophobic properties but differ in MS adhesion, was subsequently studied in the absence (Fig. 2A) or presence (Fig. 2B) of D-mannose. In the absence of D-mannose, strain 1785 (MS^+) was cleared much more rapidly than strain 3255 (MS^-). For example, at 15 min after injection, the clearance of strain 1785 was 30-fold higher than that of strain 3255. However, when mannose was present in the coinoculation mixture, the clearance of MS^+ strain 1785 was greatly inhibited and similar to that of MS^- strain 3255 (Fig. 2B). As expected, clearance of the latter strain was not significantly affected by addition of the monosaccharide.

TABLE 2. In vitro survival of *S. marcescens* strains in fresh mouse blood versus PBS

Strain	Mean (±SD) CFU (10^3)/ml after incubation[a] in:	
	PBS	Fresh mouse blood
RZ	16.6 ± 1.7	25.4 ± 2.4
3162	41.3 ± 4.8	50.9 ± 4.2
3164	74.4 ± 12.6	139 ± 24
1785	67.0 ± 1.4	80.0 ± 41
3255	33.1 ± 5.3	106 ± 38

[a] After 1 h of incubation at 37°C.

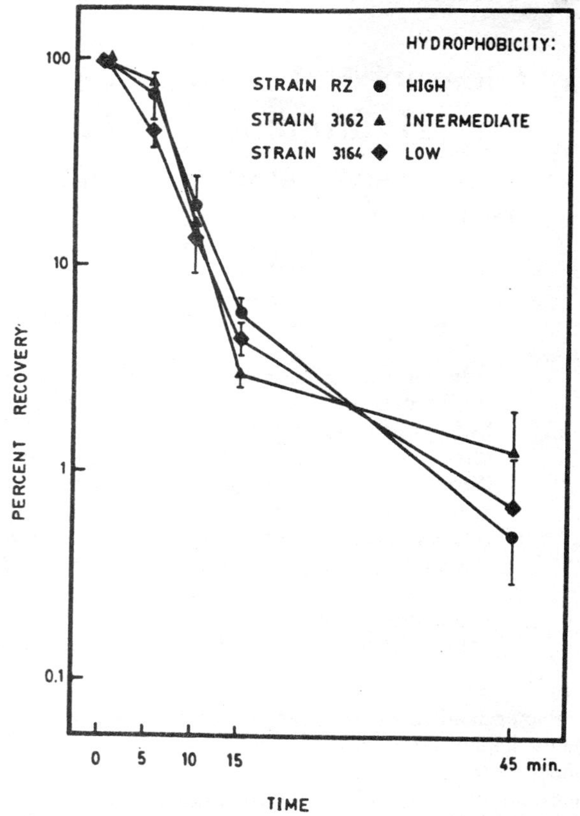

FIG. 1. Effect of cell surface hydrophobicity on clearance from blood of *S. marcescens*. Strains RZ, 3162, and 3164 were grown, washed, and suspended in PBS as described in Materials and Methods. Equal volumes of bacterial suspensions were combined to obtain the inoculation mixture. At various times after inoculation, blood samples were taken, diluted first into tap water and then into PBS, and plated. The results are expressed as the mean recovery percentages (± the standard deviation) of strains RZ, 3162, and 3164 as a function of time. The time zero values were calculated by estimating the total blood volume as 8% (vol/wt) of the average weight (20 g) of the mice used (5).

To determine whether the observed inhibition by D-mannose was a general sugar effect, additions of D-mannose and D-glucose were compared (Table 3). D-Glucose did not appear to inhibit rapid clearance of MS^+ strain 1785 from blood as did D-mannose. For example, 10 min after inoculation, only 2.7% of the MS^+ cells could be recovered when glucose was present, as opposed to 32% when mannose was present. As expected, MS^- strain 3255 was cleared at the same rate whether mannose or glucose was present.

DISCUSSION

Bacterial hydrophobicity has emerged in recent years as an important factor in a wide array of adhesion and adhesion-related phenomena (14), including hemagglutination (4)

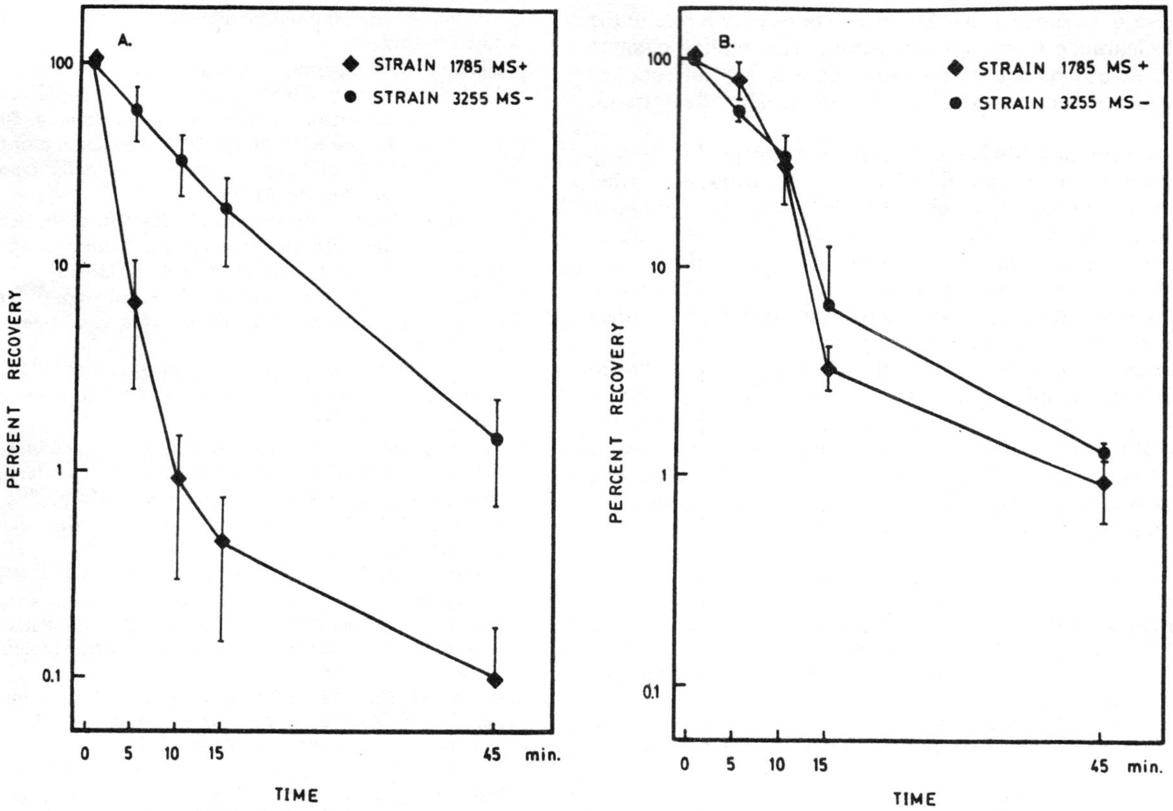

FIG. 2. Clearance of MS$^+$ versus MS$^-$ *S. marcescens* from blood in the absence or presence of D-mannose. MS$^+$ strain 1785 and MS$^-$ 3255 were grown and prepared as described in Materials and Methods. Equal volumes of the two bacterial suspensions were combined to obtain the inoculation mixture. At various time intervals after inoculation, blood samples were taken, diluted into PBS, and plated. The results are expressed as the mean recovery percentages (± the standard deviation) of strains 1785 and 3255 as a function of time. (A) Results obtained without mannose. The differences between the recoveries of strains 1785 and 3255 were statistically significant ($P < 0.05$) at 5 and 10 min after injection. (B) Results obtained with D-mannose in the inoculation mixture. Both experiments used the same bacterial suspensions and were carried out simultaneously. The time zero values were calculated by estimating the total blood volume as 8% (vol/wt) of the average weight (20 g) of the mice used (5).

and phagocytosis (11, 15). Stereospecific, lectinlike interactions have also been shown to mediate associations of many bacterial species with host cells (1–3, 5, 7, 9), including phagocytosis (2) and clearance from blood (3, 5, 9). It was thus of interest to see whether either or both of these mechanisms mediate clearance from blood in *S. marcescens*, a microorganism which has been increasingly implicated in nosocomial infections (10, 16) and which can simultaneously exhibit both MS$^+$ and hydrophobic surface properties (13).

To assess the potential role of cell surface hydrophobicity, the clearances from blood of hydrophobic wild-type strain RZ, mutant 3162, which is partially deficient in hydrophobic surface properties, and nonhydrophobic mutant 3164 were compared. The different colony morphologies of the wild-type and mutant cells (Table 1) enabled us to coinoculate them into test animals. Despite the large differences in cell surface hydrophobicity, they did not differ in their respective clearance rates. The possibility that pigmentation is involved in clearance from blood can be ruled out, since nonpigmented strain 3162 was cleared in a manner similar to that of the two pigmented strains, RZ and 3164.

To study the potential role of MS adhesins, two clinical isolates with similar hydrophobic properties but differing in MS properties were compared. One pigmented and one nonpigmented isolate were chosen so that they could be coinoculated and subsequently distinguished. MS$^+$ strain 1785 was cleared much more rapidly than MS$^-$ strain 3255. To further establish a role for MS interactions in the rapid clearance of strain 1785, the two strains were also coinoculated in the presence of D-mannose. Whereas clearance of strain 3255 (MS$^-$) was not appreciably affected, mannose greatly inhibited the clearance of strain 1785 (MS$^+$). Addition of D-glucose did not similarly inhibit the rapid clearance of MS$^+$ strain 1785, a further indication of the specificity of the mannose-mediated inhibition.

TABLE 3. Inhibition of rapid clearance from blood by D-mannose but not by D-glucose[a]

Time (min) after injection	Mean (±SD) % recovery of[b]:			
	Strain 1785 (MS$^+$)		Strain 3255 (MS$^-$)	
	D-Glucose	D-Mannose	D-Glucose	D-Mannose
5	19 ± 5	50 ± 2	66 ± 13	61 ± 10
10	2.7 ± 2	32 ± 10	29 ± 16	33 ± 7
45	0.37 ± 0.16	4.5 ± 0.8	3.6 ± 1.2	4.3 ± 0.8

[a] The clearance of a mixture of strains 1785 (MS$^+$) and 3255 (MS$^-$) from blood was studied, as described in the legend to Fig. 2, in the presence of either D-mannose or D-glucose (180 mg/ml).

[b] Results are expressed as mean percent recovery to the initial inoculum. The differences between the recoveries of strains 1785 and 3255 in the presence of glucose were statistically significant ($P < 0.05$) at 5 and at 45 min after injection.

These data suggest that MS interactions play a role in the rapid clearance from blood observed. This finding concurs with previous reports of the importance of MS interactions in clearance from blood of other gram-negative bacteria (3, 5, 9).

In a previous study, cell surface hydrophobicity was observed in all 14 clinical isolates of *S. marcescens* tested (13). Nevertheless, hydrophobicity does not appear to play a significant role in the clearance of *S. marcescens* from blood in mice under the conditions studied. These data do not rule out a role for cell surface hydrophobicity in host colonization by *S. marcescens*, e.g., via the gastrointestinal tract. Studies in this direction are currently in progress.

S. marcescens is a common environmental microorganism which has recently been cited in a wide variety of nosocomial infections (10, 16). Treatment is often difficult because of the resistance of clinical strains to many antibiotics (10). Clarification of the roles of various adhesins and adhesion mechanisms in host colonization by *S. marcescens* may thus be of potential therapeutic benefit.

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LITERATURE CITED

1. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325–340.
2. **Blumenstock, E., and K. Jann.** 1982. Adhesion of piliated *Escherichia coli* strains to phagocytes: differences between bacteria with mannose-sensitive pili and those with mannose-resistant pili. Infect. Immun. **35**:264–269.
3. **Friedman, R. L., and R. J. Moon.** 1977. Hepatic clearance of *Salmonella typhimurium* in silica-treated mice. Infect. Immun. **16**:1005–1012.
4. **Garber, N., N. Sharon, D. Shohet, J. S. Lam, and R. J. Doyle.** 1985. Contribution of hydrophobicity to hemagglutination reactions of *Pseudomonas aeruginosa*. Infect. Immun. **50**:336–337.
5. **Leunk, R. D., and R. J. Moon.** 1982. Association of type 1 pili with the ability of livers to clear *Salmonella typhimurium*. Infect. Immun. **36**:1168–1174.
6. **Mirelman, D., G. Altmann, and Y. Eshdat.** 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. J. Clin. Microbiol. **11**:328–331.
7. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. Infect. Immun. **22**:247–254.
8. **Ofek, I., E. Whitnack, and E. H. Beachey.** 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. **154**:139–145.
9. **Perry, A., and I. Ofek.** 1984. Inhibition of blood clearance and hepatic tissue binding of *Escherichia coli* by liver lectin-specific sugars and glycoproteins. Infect. Immun. **43**:257–262.
10. **Sleigh, J. D.** 1983. Antibiotic resistance in *Serratia marcescens*. Br. Med. J. **287**:1651–1653.
11. **Speert, D. P., B. A. Loh, D. A. Cabrel, and I. E. Salit.** 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. **53**:207–212.
12. **Rosenberg, M.** 1984. Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. J. Bacteriol. **160**:480–482.
13. **Rosenberg, M., Y. Blumberger, H. Judes, R. Bar-Ness, E. Rubinstein, and Y. Mazor.** 1986. Cell surface hydrophobicity of pigmented and nonpigmented clinical *Serratia marcescens* strains. Infect. Immun. **51**:932–935.
14. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. Adv. Microb. Ecol. **9**:353–393.
15. **van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32**:19–40.
16. **Yu, V. L.** 1979. *Serratia marcescens*: historical perspective and clinical review. N. Engl. J. Med. **300**:887–893.

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Increased Cell Surface Hydrophobicity of a *Serratia marcescens* NS 38 Mutant Lacking Wetting Activity

RONIT BAR-NESS,[1] NAFTALI AVRAHAMY,[1] TOHEY MATSUYAMA,[3] AND MEL ROSENBERG[1,2]*

The Maurice and Gabriela Goldschleger School of Dental Medicine,[1] and Department of Human Microbiology,[2] Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel, and Department of Bacteriology, Niigata University School of Medicine, 1-757 Asahimachidori, Niigata 951, Japan[3]*

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The cell surface hydrophobicity of *Serratia marcescens* appears to be an important factor in its adhesion to and colonization of various interfaces. The cell surface components responsible for mediating the hydrophobicity of *S. marcescens* have not been completely elucidated, but may include prodigiosin and other factors. In the present report we have investigated the potential role of serratamolide, an amphipathic aminolipid present on the surfaces of certain *S. marcescens* strains, in modulating cell surface hydrophobicity. The hydrophobic properties of a serratamolide-producing strain (NS 38) were compared with those of a serratamolide-deficient mutant (NS 38-9) by monitoring the kinetics of adhesion to hexadecane. Serratamolide production was monitored by thin-layer chromatography and the wetting activity of washed-cell suspensions on polystyrene. Wild-type NS 38 cells were far less hydrophobic than the serratamolide-deficient mutant cells were; the removal coefficients were 48 min^{-1} for the mutant, as compared with only 18 min^{-1} for the wild type. The data suggest that the presence of serratamolide on *S. marcescens* cells results in a reduction in hydrophobicity, presumably by blocking hydrophobic sites on the cell surface.

Serratia marcescens is a common environmental microorganism of considerable historical interest (2) which has been increasingly implicated as a primary pathogen in numerous human infections (14, 19). The pronounced hydrophobic surface characteristics of *S. marcescens* were reported by Mudd and Mudd in 1924 (9) and have been studied in various laboratories during recent years (3, 4, 10, 11, 15). Cell surface hydrophobicity in *Serratia* strains enables them to concentrate at water surfaces, providing them with the possible advantages of nutrient scavenging (3, 15) and dispersal in jet bubbles (1). Recently, a role for cell surface hydrophobicity in initial adhesion and host colonization by clinical *S. marcescens* strains was proposed, based on the observation that all 14 clinical isolates examined exhibited hydrophobic surface properties (11).

The molecular moieties which may promote cell surface hydrophobicity in *Serratia* strains include prodigiosin (1, 3–5, 15) and a 70-kilodalton outer surface protein (R. Bar-Ness and M. Rosenberg, submitted for publication). In a recent report, Matsuyama et al. (7) suggested that serratamolide may also be involved in modulating cell surface hydrophobicity. Serratamolide, an amphipathic aminolipid present on the surfaces of certain *Serratia* strains, was first characterized by Wasserman et al. (16, 17) and was recently shown to be a potent wetting agent (7). In the work described in this report we have compared the hydrophobic surface properties of a serratamolide-producing *S. marcescens* strain with those of a mutant deficient in serratamolide production. Hydrophobicity was determined by studying the kinetics of adhesion of hexadecane, a technique which allows highly reproducible, quantitative measurements (6, 13). The data suggest that the presence of serratamolide results in a large reduction in cell surface hydrophobicity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. marcescens* NS-38 (ATCC 13880) is a pigmented serratamolide-producing strain (7); strain NS 38-9, obtained and characterized by Matsuyama et al. (8), selected for its lack of wetting-agent activity, is deficient in serratamolide production.

Bacteria were inoculated as lawns on peptone glycerol (PG) agar (5 g of Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 10 ml of glycerol [Sigma Chemical Co., St. Louis, Mo.], 12 g of agar [Difco], distilled water to 1,000 ml) and grown for 3 days at 30 to 38°C as indicated.

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by measuring the kinetics of adhesion to hexadecane (6), essentially as described by Sharon et al. (13). Bacteria were washed twice and suspended in phosphate buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, distilled water to 1,000 ml [pH 7.2]) to an initial absorbance (A_0) of 0.95 to 1.05, as measured in a Uvikon 710 spectrophotometer (light path, 1 cm; Kontron, Zurich, Switzerland) at 400 nm. Bacterial suspensions (1.6 ml) were added in quadruplicate to semimicro disposable polystyrene cuvettes (4 ml; Rudolf Brand, Wertheim, Federal Republic of Germany), and hexadecane was then added as indicated. Mixtures were vortexed at room temperature on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuque, Iowa) for 5-s intervals. After each mixing period, the phases were allowed to separate and the absorbance of the lower, aqueous phase (A_t) was measured. The rate of adhesion, designated the removal rate, R, was calculated as log ($A_t \cdot 100/A_0$). Linear regression was used to plot the results.

For endpoint measurement of bacterial adhesion to hexadecane, 25 μl of hexadecane was added to 1.6 ml of bacterial suspension as described above. The mixtures were then vortexed for 30-s periods until no further decrease in turbidity was observed.

Thin-layer chromatography. Bacteria were grown at 30 or 38°C on PG agar for 3 days (7). To examine the wetting agent, we washed wet bacterial mass from the agar surface in

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* Corresponding author.

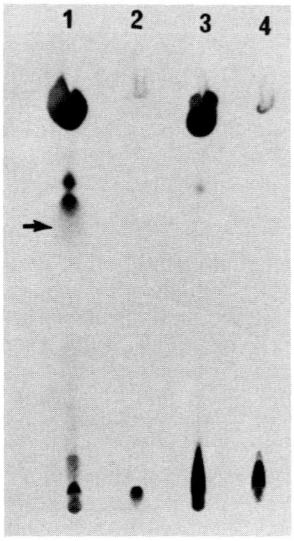

FIG. 1. Thin-layer chromatograms of lipids extracted from *S. marcescens* cells. The lipid possessing wetting activity (corresponding to serratamolide) is indicated by the arrow. Lanes: 1, strain NS 38 grown at 30°C; 2, strain NS 38 grown at 38°C; 3, mutant strain NS 38-9 grown at 30°C; 4, mutant strain NS 38-9 grown at 38°C.

20 ml of phosphate-buffered saline (8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, distilled water to 1,000 ml [pH 7.1]). Following a 15-min centrifugation (8,600 × *g*), the cell pellet was extracted with 10 volumes of ethanol. After removal of sediments by centrifugation as above and removal of the ethanol by evaporation, the dry material was further extracted with chloroform-methanol (2:1, vol/vol). The extracts were examined on a thin-layer chromatography plate of silica gel (DC-plastikfolien Kieselgel 60 F254; E. Merck AG, Darmstadt, Federal Republic of Germany) in a solvent system of chloroform-methanol-acetone-acetic acid (90:10:6:1 by volume). For detection of components the plate was sprayed with 50% H_2SO_4 in ethanol and heated briefly at ca. 200°C.

RESULTS

S. marcescens NS 38, grown on PG agar at 30°C, produced cell-associated serratamolide (Fig. 1, lane 1). However, when the cells were grown at 38°C serratamolide was no longer detected (lane 2). Wetting-agent-deficient mutant NS 38-9 did not produce serratamolide following growth at either temperature (lanes 3 and 4). These results are in agreement with previous data of Matsuyama et al. (7, 8).

Previous investigations have shown that pigment (prodigiosin) production is temperature dependent (18). Both strains produced pigment (prodigiosin) when grown at 30°C but not at 38°C (Fig. 1).

Figure 2 illustrates the spreading of cell suspensions on polystyrene. Following growth at 30°C, a 10-μl droplet containing a wild-type NS 38 cell suspension (optical density at 400 nm, ca. 7.0) spread over the hydrophobic test surface, an indication of wetting activity (Fig. 2A, drop 1), whereas a droplet containing mutant NS 38-9 cells exhibited no wetting activity whatsoever (Fig. 2A, drop 2). Neither strain exhibited wetting activity after growth at 38°C (Fig. 2B) (7, 8).

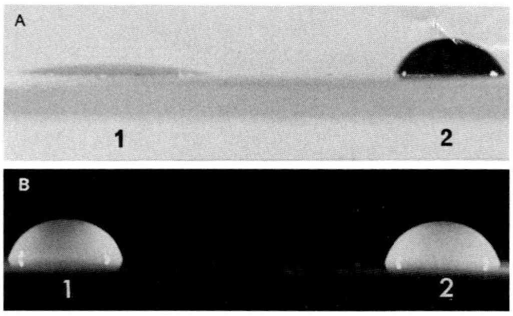

FIG. 2. Wetting activity of bacterial suspension on polystyrene. The wetting activity of washed suspensions of *S. marcescens* grown at 30°C (panel A) or 38°C (panel B) was demonstrated by placing 10-μl droplets on untreated polystyrene disks. (A) Drop 1, strain NS 38; drop 2, mutant NS 38-9. (B) Drop 1, strain NS 38; drop 2, mutant NS 38-9.

To compare the hydrophobic surface properties of the strains, we used adhesion to hexadecane. The kinetics of adhesion (removal rate) of cells grown at 30°C to various hydrocarbon volumes are summarized in Fig. 3 to 5. Wild-type NS 38 cells (Fig. 3) were far less hydrophobic than cells of the corresponding serratamolide-deficient mutant were (Fig. 4). For example, whereas 84 ± 3% of the mutant cells were removed by 25 μl of hexadecane after 25 s of vortexing, only 34 ± 4% of the wild-type cells were removed under these conditions. Similarly, 5 μl of hexadecane removed 24 ± 2% of the mutant cells after 25 s of vortexing, as compared with 2 ± 1% of the wild-type cells.

When the adhesion (removal) rate is plotted as a function

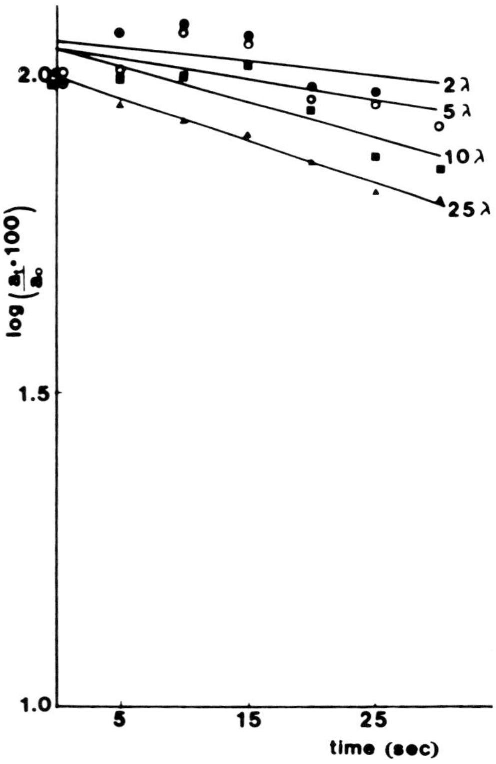

FIG. 3. Kinetics of adhesion of strain NS 38 to hexadecane. Following growth at 30°C, washed bacterial suspensions (1.6 ml) were mixed with hexadecane volumes (as indicated) for 5-s intervals as described in Materials and Methods. Results are presented as log A_r/log A_0 as a function of mixing time.

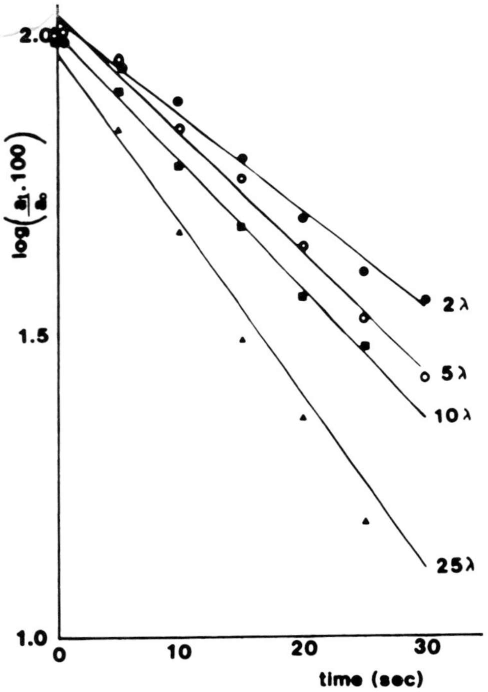

FIG. 4. Kinetics of adhesion of mutant NS 38-9 to hexadecane. Following growth at 30°C, washed bacterial suspensions (1.6 ml) were mixed with hexadecane volumes (as indicated) for 5-s intervals as described in Materials and Methods. Results are presented as log A_t/log A_0 as a function of mixing time.

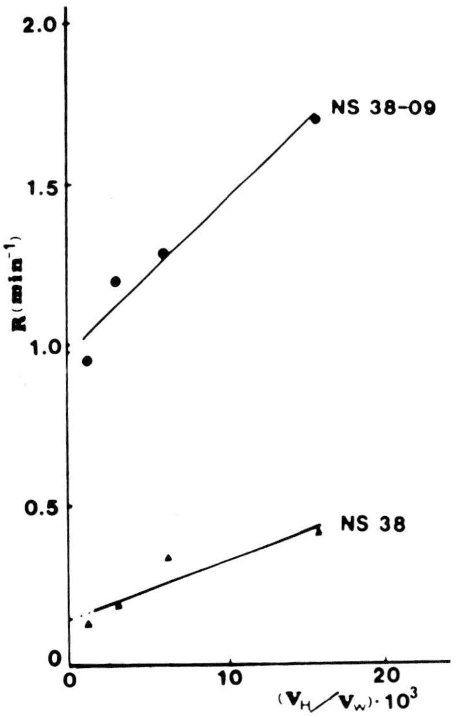

FIG. 5. Removal rate as a function of hexadecane-to-water volume ratio (v_H/v_W). Removal rates of strain NS 38 and mutant NS 38-9 (following growth at 30°C) were obtained from the slopes of the adhesion kinetics plots (Fig. 3 and 4). The removal rates are plotted as a function of the hexadecane-to-water volume ratio; the slopes yield the removal coefficient values for the two strains.

of the hexadecane-to-water volume ratio (Fig. 5), a slope is obtained which denotes the removal coefficient, a quantitative indication of the affinity of the cells for the hydrocarbon/water interface (13). The removal coefficients were 18 min^{-1} for the wild-type cells, as opposed to 48 min^{-1} for the wetting-agent-deficient mutant cells. Correlation coefficients for plots of removal rates and removal coefficients were significant in all cases ($P < 0.025$).

Following growth at 38°C, no adhesion was evident in either strain, as measured by the endpoint procedure. Previous investigations have shown that cell surface hydrophobicity is lost after growth at this temperature (11).

DISCUSSION

The data suggest that loss of serratamolide production in a mutant of *S. marcescens* (NS 38-9) is accompanied by a large increase in cell surface hydrophobicity. It may be inferred that when this amphipathic compound is present on the wild-type cell surface, it binds to hydrophobic sites, burying its nonpolar moieties toward the cell surface and exposing its polar sites into the bulk aqueous phase. Although we cannot completely rule out the possibility that the mutant strain lacks components in addition to serratamolide, it may be noted that outer surface protein profiles of mutant and wild-type cells were identical on sodium dodecyl sulfate-polyacrylamide gels (results not shown).

Recent studies of bacterial cell surface hydrophobicity suggest that hydrophobic properties are often determined by an interplay of "hydrophobins" (hydrophobicity-promoting components) and "hydrophilins" (hydrophobicity-reducing moieties), which coexist on the cell surface (12). Various investigators have suggested that the pigment prodigiosin acts as a hydrophobin in various *Serratia* cells (1, 3, 4, 11, 15). A 70-kilodalton outer surface protein may also act as a hydrophobin in *S. marcescens* (Bar-Ness and Rosenberg, submitted). The data presented here suggest that serratamolide may act as a hydrophilin, counteracting the hydrophobicity conferred by these components. In this context it is of interest that *S. marcescens* RZ exhibits high cell surface hydrophobicity, despite its production of a wetting agent, whereas its semihydrophobic nonpigmented mutant, strain 3162, is devoid of wetting activity (10; R. Bar-Ness and M. Rosenberg, unpublished data). It may be argued that the partial hydrophobicity exhibited by this mutant is the net result of loss of a hydrophobin (prodigiosin), counteracted to some extent by the absence of a hydrophilin (serratamolide). This hypothesis is currently under investigation.

Modulation of cell surface hydrophobicity has been suggested as a possible factor in host colonization by clinical *S. marcescens* isolates (11). The possibility that serratamolide plays a role in such a phenomenon warrants further study.

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LITERATURE CITED

1. **Blanchard, D. C., and L. D. Syzdek.** 1978. Seven problems in bubble and jet drop researches. Limnol. Oceanogr. **23:**389–400.
2. **Doyle, R. J., and N. C. Lee.** 1986. Microbes, warfare, religion and human institutions. Can. J. Microbiol. **32:**193–200.

3. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T. A. Stenstrom.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion to an air-water interface. Arch. Microbiol. **131:**308–312.
4. **Hermansson, M., S. Kjelleberg, and B. Norkrans.** 1979. Interaction of pigmented wild type and pigmentless mutant of *Serratia marcescens* with lipid surface film. FEMS Microbiol. Lett. **6:**129–133.
5. **Kefford, B., S. Kjelleberg, and K. C. Marshall.** 1982. Bacterial scavenging utilization of fatty acids localized at a solid-liquid interface. Arch. Microbiol. **133:**257–260.
6. **Lichtenberg, D., M. Rosenberg, N. Sharfman, and I. Ofek.** 1985. A kinetic approach to bacterial adherence to hydrocarbon. J. Microbiol. Methods **4:**141–146.
7. **Matsuyama, T., M. Fujita, and I. Yano.** 1985. Wetting agent produced by *Serratia marcescens*. FEMS Microbiol. Lett. **28:**125–129.
8. **Matsuyama, T., M. Sogawa, and I. Yano.** 1987. Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. Appl. Environ. Microbiol. **53:**1186–1188.
9. **Mudd, S., and E. B. H. Mudd.** 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. J. Exp. Med. **40:**633–645.
10. **Rosenberg, M.** 1984. Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. J. Bacteriol. **160:**480–482.
11. **Rosenberg, M., Y. Blumberger, H. Judes, R. Bar-Ness, E. Rubinstein, and Y. Mazor.** 1986. Cell surface hydrophobicity of pigmented and nonpigmented *Serratia marcescens* strains. Infect. Immun. **51:**932–935.
12. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. Adv. Microb. Ecol. **9:**353–393.
13. **Sharon, D., R. Bar-Ness, and M. Rosenberg.** 1986. Measurement of the kinetics of bacterial adherence to hexadecane in polystyrene cuvettes. J. Microbiol. Methods **4:**141–146.
14. **Sleigh, J. D.** 1983. Antibiotic resistance in *Serratia marcescens*. Br. Med. J. **287:**1651–1653.
15. **Syzdek, L. D.** 1982. Concentration of *Serratia* in the surface microlayer. Limnol. Oceanogr. **26:**961–964.
16. **Wasserman, H. H., J. J. Keggi, and J. E. McKeon.** 1961. Serratamolide, a metabolic product of *Serratia*. J. Am. Chem. Soc. **83:**4107–4108.
17. **Wasserman, H. H., J. J. Keggi, and J. E. McKeon.** 1962. The structure of serratamolide. J. Am. Chem. Soc. **84:**2978–2982.
18. **Williams, R. P., and S. M. H. Qadri.** 1980. The pigment of *Serratia*, p. 31–75. *In* A. von Graevenitz and S. J. Rubin (ed.), The genus *Serratia*. CRC Press, Inc., Boca Raton, Fla.
19. **Yu, V. L.** 1979. *Serratia marcescens*—historical perspective and clinical review. N. Engl. J. Med. **300:**887–891.

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A Novel Replica Technique for Localization of Caries-Associated Bacteria on Tooth Surfaces: Development and Initial Experience

(Short Communication; with 1 color plate)

M. Rosenberg[a, b], *E. Weiss*[a], *I. Eli*[a]

[a] Maurice and Gabriela Goldschleger School of Dental Medicine, and
[b] Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Key Words. Caries risk · Diagnosis · Replica technique · *Streptococcus mutans*

In the present report, a novel replica technique for localizing caries-associated bacteria on tooth surfaces is described. The method is highly suitable for dental clinics, can be performed by semi-skilled personnel and may have applications in caries prediction, diagnosis and management, as well as in related lines of research. While at present several tests for estimation of caries-associated bacteria in saliva exist [Kohler and Bratthall, 1979; Matsukubo et al., 1981; Newbrun, 1983; Nikoforuk, 1985], such methods have found only limited clinical use, presumably since they do not attempt to locate the actual sites of caries activity [Nikoforuk, 1985]. The primary aim of this study was thus to develop a simple replica technique which could identify the location of caries-associated microorganisms on tooth surfaces. Our initial premise was that adherent caries-associated bacteria can be transferred from tooth surfaces onto certain impressionable substances. Initial experiments, using agar overlay techniques similar to those previously reported [Sakamaki and Bahn, 1968; Beavers et al., 1984] demonstrated that selective growth of caries-associated streptococci could be observed at distinct sites using certain impression materials. It was clear, however, that such an approach is far too cumbersome to be envisioned for routine use in dental clinics. A technique was subsequently sought which would enable localized outgrowth using a liquid, rather than solid, growth medium.

The strategy employed was to incorporate a necessary growth nutrient within the solid matrix, and leave it out of the surrounding liquid medium. In this manner, growth would be restricted to the solid/liquid interface, and contamination and spreading via the bulk aqueous phase avoided. To this end, the main carbon and energy source, sucrose, was incorporated into the solid matrix and left out of the corresponding liquid medium.

Formulations of the solid matrix and liquid outgrowth medium have been previously specified [Rosenberg, Eli, Weiss, US Patent Appl. Serial No. 06/770201]; samples of solid matrix and liquid medium may be requested from the authors. The solid

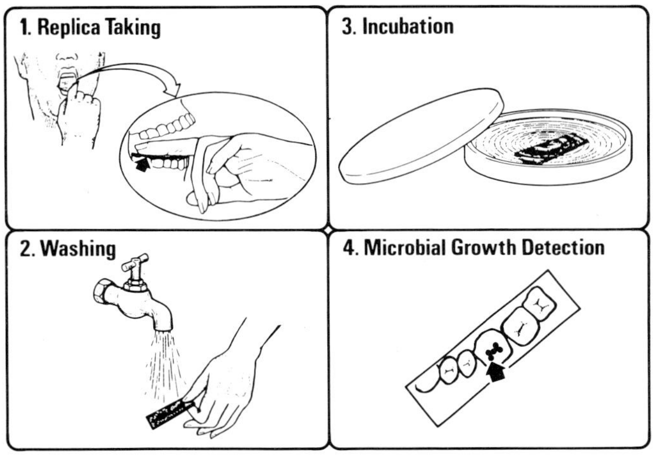

Fig. 1. Stages of the replica technique. The matrix material is pressed against the tooth surface to be tested (1), and then washed briefly in tap water (2). Following overnight incubation in the sucrose-free medium (3), loci of growth can be directly observed (4).

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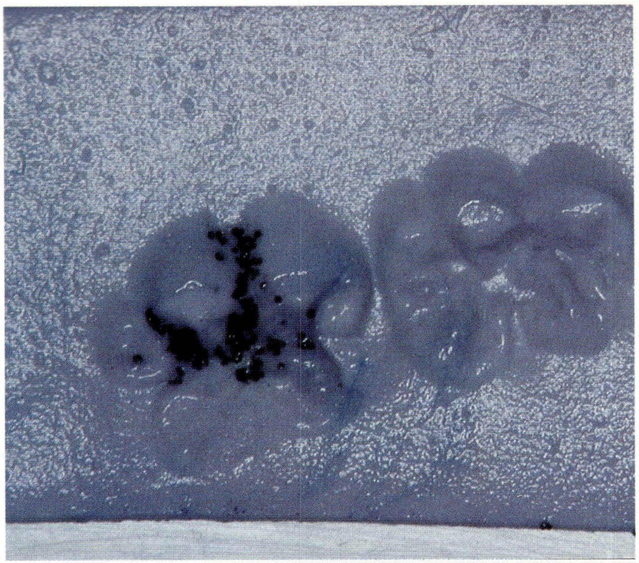

Fig. 2. Replica of occlusal surfaces. Growth can be readily observed on the replica imprint corresponding to certain occlusal fissures on second molar, whereas adjacent first molar appears free of growth.

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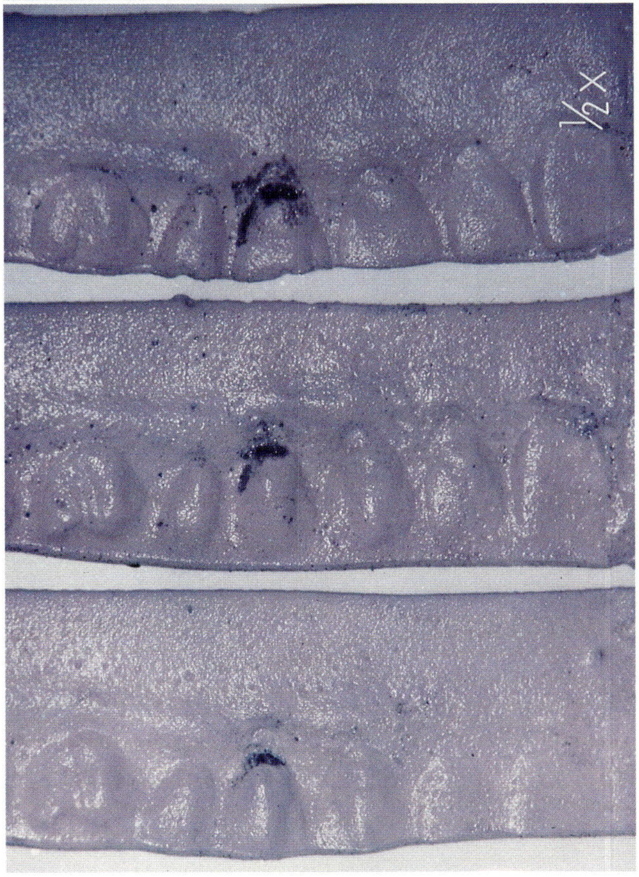

Fig. 4. Replicas of cervical lesion. Replicas were taken of the area of the cervical lesion shown in figure 3 at 9.45 h (bottom replica), 12.15 h (center) and 14.00 h (top). Note that the extent of growth corresponding to the area of the lesion increases with time of day.

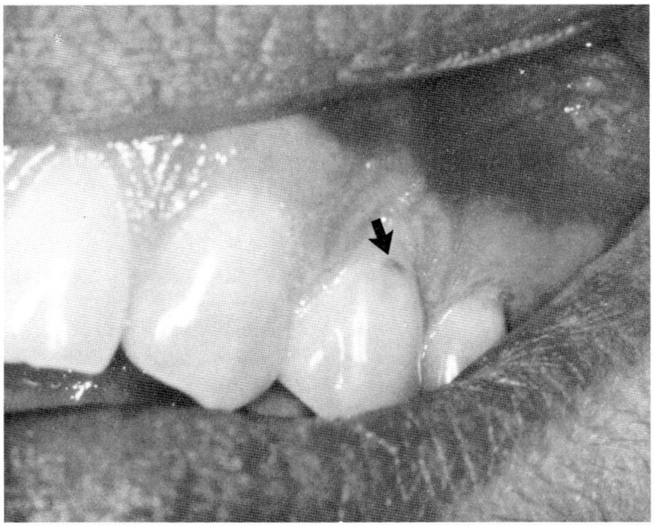

Fig. 3. Cervical lesion (arrow) in a 27-year-old female.

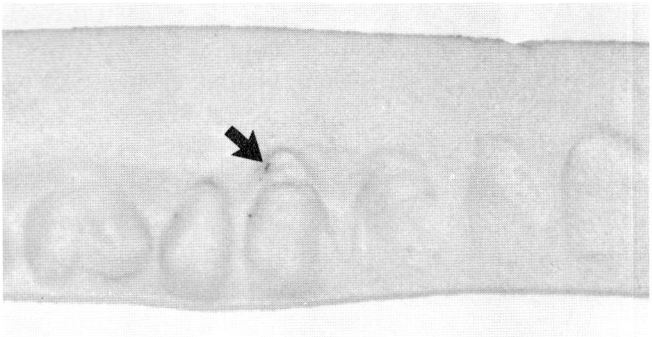

Fig. 5. Following prophylactic treatment of the cervical lesion, corresponding growth is no longer evident.

impression matrix was comprised primarily of sucrose and a commercial gum base. The liquid medium was composed of tryptose, proteose peptone, trypan blue, gentian violet and potassium tellurite, all obtained from Difco (Detroit, Mich.). Addition of bacitracin (0.5 units/ml, Sigma, St. Louis, Mo.) limited outgrowth almost exclusively to *Streptococcus mutans*, a bacterium highly associated with both occlusal [Meiers et al., 1982], cervical and root caries development [Ellen et al., 1985]. For example, in a typical experiment, an area of outgrowth was subcultured onto mitis salivarius agar; among 160 colonies examined, 154 (96%) had typical *S. mutans* morphology [Portnoy et al., unpublished data].

Figure 1 summarizes the main stages of the technique. An imprint is obtained by pressing the matrix against the tooth surfaces to be sampled. The solid matrix is then washed for several seconds in tap water to remove nonadherent cells and saliva which may interfere. The matrices are subsequently placed in the sucrose-free liquid outgrowth medium and incubated at about 35°C. Following overnight incubation, the matrices are removed from the liquid medium and examined directly for outgrowth (stained dark blue by the chromogenic agents). As shown in figure 2, growth corresponding to specific occlusal fissures can be readily observed.

Figure 3 shows a cervical lesion in a 27-year-old female. Outgrowth corresponding exclusively to the locus of the lesion was consistently observed on the buccal replica, increasing as a function of the time of day (fig. 4). Following a single prophylactic application of a fluoride gel (Elmex, Teva, Israel), growth was not detected (fig. 5); growth at the site reappeared within about 1 week. Whereas extensive additional testing is required to determine the potential clinical applications of the described technique, several possible uses may be envisioned:

(1) Diagnosis of occlusal caries. Current clinical tests for occlusal caries involve a mechanical examination using a dental explorer which is subjective, error-prone [Glass et al., 1983; Kamen and Schmee, 1974] and potentially destructive [Ekstrand and Thylstrup, 1985; van der Laan-van Dorp et al., 1985], especially in diagnosis of 'sticky fissures' where decay is not obvious. Thus, the replica technique may conceivably provide a supplementary indication of the caries risk at the sites in question.

(2) Guide for occlusal sealant application. Occlusal sites which appear healthy in a clinical examination, but exhibit outgrowth on the replica may indicate priority for sealant treatment.

(3) Cervical and root caries treatment. The replica technique may enable identification of the caries risk of exposed root and cervical surfaces, and enable follow- up on treatment regimens. This may be of special importance in cases of recessions following periodontal surgery.

(4) Caries prediction. The method may be useful to predict caries activity in young children, based on the extent of outgrowth observed on replicas taken of smooth tooth surfaces [Newbrun, personal commun.].

(5) Testing of anticaries agents. The possibility of monitoring incipient caries development may be useful in evaluation of various preventive agents, such as topical fluoride gels and antibacterial mouthrinses.

(6) Testing of restorative materials. The technique may be employed for in vivo evaluation of restorative materials for their ability or inability to harbour caries-associated microbial growth, primarily along margins of various types of restorations.

Several of these potential applications are currently under investigation.

Acknowledgements

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References

Beavers, F.P.P.; Wilson, M.; Ivanyi, L.; Hobkirk, J.A.: Comparison of salivary counts and palatal imprint cultures in patients with denture stomatitis. J. dent. Res. *63:* 519 (1984).

Ellen, R.P.; Banting, D.W.; Fillery, E.D.: Longitudinal microbiological investigation of a hospitalized population of older adults with a high root surface caries risk. J. dent. Res. *64:* 1377–1381 (1985).

Ekstrand, K.; Thylstrup, A.: A light microscopic study of the effect of probing in occlusal fissures. Caries Res. *20:* 151 (1986).

Glass, R.L.; Peterson, J.K.; Bixler, D.: The effects of changing caries prevalence and diagnostic criteria on clinical caries trials. Caries Res. *17:* 145–151 (1983).

Kamen, A.; Schmee, J.: Diagnostic errors and multiple examiners in anticariogenic studies. J. dent. Res. *53:* 1500 (1974).

Kohler, B.; Bratthall, D.: Practical method to facilitate estimation of *Streptococcus mutans* levels in saliva. J. clin. Microbiol. *9:* 584–588 (1979).

Laan-van Dorp, C.S.E. van der; Exterkate, R.A.M.; Ten Cate, J.M.: The effect of probing on fissure caries progression. Caries Res. *20:* 151 (1986).

Matsukubo, T.; Ohta, K.; Maki, Y.; Takeuchi, M.; Takazoe, I.: A semi-quantitative determination of *Streptococcus mutans* using its adherent ability in a selective medium. Caries Res. *15:* 40–45 (1981).

Meiers, J.C.; Wirthlin, M.R.; Shklair, I.L.: A microbiological analysis of human early carious and non-carious fissures. J. dent. Res. *61:* 460–462 (1982).

Newbrun, E.: Caries activity tests; in Cariology, pp. 256–273 (Williams & Wilkins, Baltimore 1983).

Nikoforuk, G.: Monitoring caries activity; in Understanding Dental Caries, vol. 2, Prevention, basic and clinical aspects, pp. 225–242 (Karger, Basel 1985).

Sakamaki, S.T.; Bahn, A.N.: Effect of orthodontic banding on localized oral lactobacilli. J. dent. Res. *47:* 275–279 (1968).

M. Rosenberg
The Maurice and Gabriela Goldschleger School
of Dental Medicine
Tel-Aviv University
Ramat-Aviv 69978
Tel-Aviv (Israel)

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SALIVA-MEDIATED INHIBITION AND PROMOTION OF BACTERIAL ADHESION TO POLYSTYRENE

ILANA ELI*, HERBERT JUDES and MEL ROSENBERG[1]

Maurice and Gabriela Goldschleger School of Dental Medicine, and [1]*Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, 69978 Tel-Aviv, Israel*

(*Received 3 February, 1988*)

A simple model system was developed in order to study the effect of saliva on bacterial adhesion to a hydrophobic model surface, polystyrene. Washed bacterial suspensions of *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) and *Serratia marcescens* RZ were mixed with serial dilutions of clarified saliva, and the mixtures incubated in wells of untreated polystyrene microtiter plates. Adhesion was recorded spectrophotometrically after staining of adherent cells. Saliva inhibited adhesion of *S. marcescens* RZ in a concentration-dependent manner. Of interest was the finding that saliva exerts two distinct effects on adhesion of *A. calcoaceticus* RAG-1. It inhibits adhesion at low saliva concentrations, but causes aggregation of RAG-1 cells and stimulation of adhesion at high saliva concentrations. Extraction of saliva with hexadecane, prior to the adhesion assay, brought about an almost complete loss of the saliva-mediated inhibition of adhesion in both strains. This same treatment, however, did not prevent the aggregation of RAG-1 cells and the concomitant promotion of adhesion at high saliva concentrations. Initial evidence suggests that the components responsible for saliva-mediated inhibition of adhesion to polystyrene are of low (< 15 kD) molecular weight.

KEY WORDS: *Serratia marcescens, Acinetobacter calcoaceticus,* adhesion, saliva, hydrophobicity, polystyrene.

INTRODUCTION

The role of hydrophobic interactions in mediating bacterial adhesion phenomena has gained increasing recognition in recent years. Several investigations have raised the possibility that adhesion within the oral ecosystem is promoted by non-specific, hydrophobic interactions (Rosenberg & Kjelleberg, 1986). If this is the case, then the ability of saliva to influence such adhesion mechanisms is clearly of interest. Most previous studies have sought to deal with the effect of saliva on adhesion under conditions simulating those in the oral cavity, e.g. with oral strains and substrata resembling the tooth surface, such as saliva-coated hydroxylapatite. However, in such systems, the relative contributions of specific (i.e. lectin-like) and non-specific (i.e. hydrophobic) interactions are difficult to ascertain. In order to focus on the latter, we have studied the effect of saliva on adhesion to a hydrophobic test surface (polystyrene), employing two non-oral strains (*Acinetobacter calcoaceticus* RAG-1 and *Serratia marcescens* RZ), whose avid adhesion to this substratum has previously been demonstrated (Rosenberg *et al.*, 1982; Rosenberg, 1984).

The results of the present study suggest that saliva inhibits adhesion of *S. marcescens* RZ to polystyrene in a concentration-dependent manner. However, with respect to *A. calcoaceticus* RAG-1 cells, saliva exerts two opposing mechanisms, inhibiting adhesion at low salivary concentrations and stimulating adhesion at high concentrations. The ability of saliva to inhibit adhesion of both strains can be abolished by pre-extracting the saliva with hexadecane.

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*To whom correspondence should be addressed at The Maurice and Gabriela Goldschleger School of Dental Medicine.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A. calcoaceticus RAG-1 (ATCC 31012; Rosenberg *et al.*, 1982) and *S. marcescens* RZ (Rosenberg, 1984) were employed. Cells were inoculated in 1 ml suspension:200 ml medium and grown for 18 h with shaking at 30°C in brain heart infusion broth (Difco Laboratories, Detroit, Michigan, USA). Cells were harvested by centrifugation, washed twice and suspended in phosphate buffered saline (PBS; 8·0 g NaCl, 0·2 g KCl, 1·15 g Na_2HPO_4, 0·2 g KH_2PO_4, and distilled water to 1 litre, pH 7·2) to an optical density of 6–7, as measured in a Uvikon 710 (Kontron, Zurich, Switzerland) spectrophotometer at 540 nm.

Saliva

Unstimulated whole saliva was collected from one of the authors, 1–2 h after breakfast, into a centrifuge tube and immediately clarified by centrifugation at 10 000 × **g** for 10 min. Microscopic observation ensured that the clarified saliva was free of bacterial cells and other particulate matter. Saliva was then diluted in two-fold serial dilutions with PBS, and promptly used in the adhesion or aggregation experiments.

Adhesion Assay

The adhesion assay was carried out to determine the effect of different saliva concentrations on bacterial adhesion to polystyrene. To bacterial suspension (2 ml), saliva (4 ml) or a serial dilution was added, and the mixture vortexed vigorously for 2 min. Aliquots (200 μl) were added to wells of flat-bottom, non-wettable (water contact angle *c.* 90°) polystyrene microtiter plates (Nunc, Roskilde, Denmark), and incubated for 15 min at room temperature. Plates were then rinsed thoroughly for 1 min with running water at room temperature and normal to the plate surface, in order to remove bulk phase bacteria without affecting firmly bound cells (Rosenberg, 1981); adhering bacteria were then stained with Gentian Violet (Fletcher, 1976) for 15 min. Adhesion was measured as the absorbance at 550 nm, using a Titertek Multiscan MC (Flow Laboratories, McLean, Virginia, USA) microtiter plate reader. As control, PBS (4 ml) was substituted for saliva or saliva dilution.

The effect of precoating the polystyrene surface with whole clarified saliva was also tested. Saliva aliquots (0·2 ml) were added to wells and, after 15 min incubation, washed extensively with PBS. Bacterial suspension of *S. marcescens* in PBS (see above) was then added and adhesion to saliva pre-coated wells measured (above).

The effect of pre-incubating the cells in saliva was tested by 15 min incubation, followed by washing the cells (× 3) with PBS, and the adhesion assay (above).

Aggregation Assay

In order to study aggregation of RAG-1 cells by saliva, bacterial suspensions and appropriate saliva dilutions were mixed as described above. Aliquots (1 ml) were placed in semi-micro disposable cuvettes (Brand, Wertheim-Main, West Germany) and the optical density at 540 nm recorded with a Uvikon 710 spectrophotometer (Kontron, Zurich, Switzerland). Aggregation was determined by measuring the drop in optical density due to settling of aggregates, after 18 h incubation at 4°C, as compared with that of bacterial suspensions in the absence of added saliva (Rosenberg *et al.*, 1982).

Fractionation of Saliva

Hexadecane extraction was carried out to determine whether the inhibitory components could be removed from the bulk aqueous phase by adsorption to hexadecane droplets (Rosenberg *et al.*, 1986). Saliva (2 ml) was diluted with PBS (2 ml)—the high viscosity of the undiluted saliva precluded its extraction with hexadecane without dilution—and hexadecane (2 ml) was added. The phases were then vortexed for 2 min. After phase separation, the lower aqueous phase was carefully removed, hexadecane was again added and the extraction procedure repeated. To ensure complete removal of hexadecane from the lower phase, the latter mixture was centrifuged at 5000 **g** for 5 min. The clear lower aqueous phase was then removed for adhesion and aggregation tests.

In order to determine the approximate molecular weight of the inhibitory components, dialysis of saliva was carried out in an Minicon concentration chamber B15 (15 kD cutoff) (Amicon Corp., Danvers, MA 09123, USA) at 4°C. The 10-fold concentrated saliva was then diluted to its original volume with PBS to test its effect on *S. marcescens* adhesion.

Surface Tension Measurement

In order to determine the effect of the hexadecane extraction procedure on surface tension of saliva, a Fisher Model 20 Surface Tensiometer (Pittsburgh, PA, USA), fitted with a platinum ring, was employed. Measurement of a saliva sample, diluted 1:1 with PBS, prior to and after extraction with hexadecane, was carried out at 20°C.

Protein

Protein in whole saliva and in hexadecane-extracted saliva (diluted 1:1 with PBS) was measured according to Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Results from a typical experiment testing adhesion of *S. marcescens* RZ to polystyrene as a function of salivary dilution present are shown in Fig. 1 (lower curve). Adhesion was found to decrease with increasing saliva concentrations. At a final concentration of 67% (v/v), adhesion was inhibited by 91%, as compared to the PBS control.

The saliva-mediated inhibition appeared to be due to coating of the polystyrene surface, rather than the cells themselves; pre-incubation of the cells in saliva, followed by washing and resuspension in PBS, did not lower adhesion; however, precoating of the polystyrene surface with saliva reduced adhesion by 78% (Fig. 2).

The inhibitory effect of saliva on the adhesion of *S. marcescens* to polystyrene could be abolished by extracting the saliva samples with hexadecane prior to the adhesion

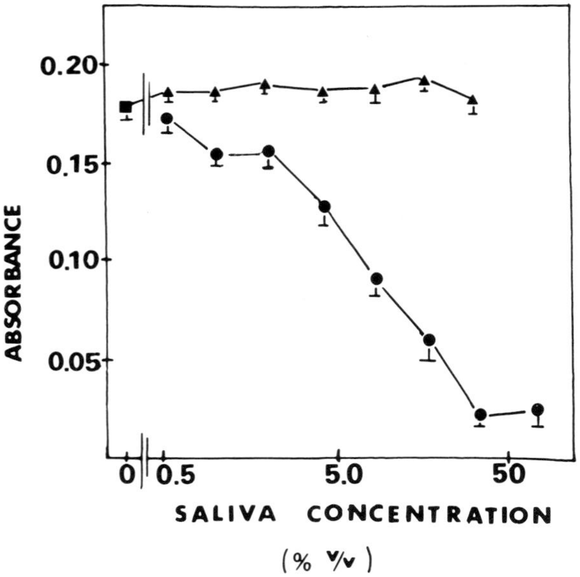

Fig. 1 Effect of unextracted and extracted saliva on adhesion of *S. marcescens* RZ to polystyrene. The lower curve (●) illustrates the effect of dilutions of unextracted saliva on adherence. The upper curve (▲) illustrates the effect of dilutions of saliva that was pre-extracted with hexadecane, as described in Materials and Methods. Each point represents the arithmetic mean ± standard deviation.

assay. Adhesion of *S. marcescens* in the presence of dilutions of saliva, pre-extracted with hexadecane, is shown in Fig. 1 (upper curve).

Extraction of saliva resulted in changes in both surface tension and protein content. Surface tension of saliva, diluted 1:1 with PBS, rose from 51·3 to 61·6 dynes·cm^{-1} after extraction; protein content fell from 0·74 to 0·56 mg·ml^{-1}.

Dialysis of saliva in a Minicon B15 chamber resulted in the loss of most of its inhibitory ability (Fig. 3), suggesting that low molecular weight (< 15 kD) components may be responsible for the observed inhibition of adhesion.

Results from a typical experiment in which the oil-degrading bacterium *A. calcoaceticus* RAG-1 was employed are shown in Fig. 4. In the presence of low saliva concentrations (lower curve), RAG-1 adhesion was inhibited, as with *S. marcescens*. For example, in the presence of 8·3% saliva (v/v), adhesion as 12% of that observed for the PBS control. However, at higher saliva concentrations, adhesion was enhanced, as compared to the no-saliva control. Microscopic examination of the bacterial suspensions revealed that RAG-1 cells were aggregated within seconds, after addition of high saliva concentrations. Quantitative measurement of the extent of aggregation of RAG-1 cells is presented in Table 1. Saliva concentrations that promoted adhesion of RAG-1 to polystyrene similarly caused significant aggregation of RAG-1 cells. In contrast, *S. marcescens* cells were not observably aggregated by any of the saliva concentrations tested.

The effect of saliva pre-extraction on adhesion of RAG-1 to polystyrene is presented in Fig. 4 (upper curve). After hexadecane extraction, saliva-mediated inhibition was no longer observed. However, high concentrations of hexadecane-extracted saliva still promoted RAG-1 adhesion.

Table 1 Saliva-mediated aggregation of RAG-1 cells[a]

Percent saliva (v/v)	*Percent aggregation*
67	60
33	62
17	35
8·3	14
4·1	12
2·1	3
1·0	0
0·5	0

[a] *A. calcoaceticus* RAG-1 cells were grown and prepared as described in the text. To 2 ml of RAG-1 cells, 4 ml of whole saliva, saliva diluted with PBS, or PBS alone (as control) were added and the mixtures vortexed vigorously for 2 min. Aliquots (1 ml were transferred to semi-micro cuvettes, and the initial turbidity was recorded at 540 nm. After 18 h incubation at 4°C, turbidity was recorded as above. Results are expressed as percent loss in turbidity, as compared to the saliva-free (PBS) control.

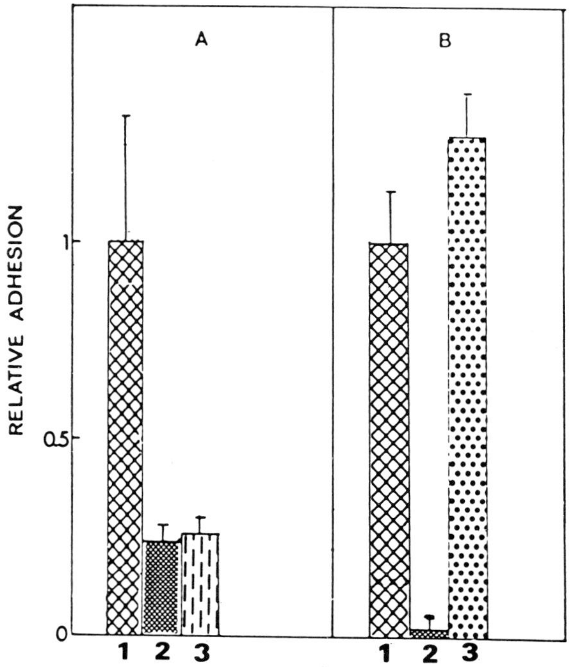

Fig. 2 Effect of saliva pre-coating of bacterial cells and polystyrene on adhesion of *S. marcescens*. **Fig. 2A** illustrates the effect of precoating the polystyrene surface with whole clarified saliva. Columns: (1) bacterial suspension, added to non-coated polystyrene wells; (2) adhesion (relative to column 1) of a two-fold concentrated bacterial suspension, diluted 1:1 with whole clarified saliva, to non-coated wells; (3) adhesion (relative to column 1) of bacterial suspension to saliva-precoated wells. **Fig. 2B** shows the effect of preincubating *S. marcescens* cells in the presence of whole clarified saliva. Columns: (1) adhesion of non-treated bacterial suspension; (2) adhesion (relative to column 1) of two-fold concentrated bacterial suspension diluted 1:1 with whole clarified saliva; (3) adhesion (relative to column 1) of saliva-preincubated cells.

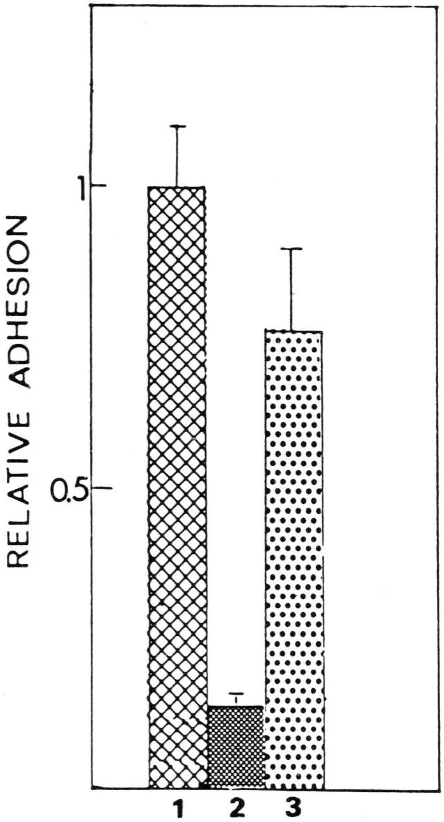

Fig. 3 Effect of dialysed saliva on adhesion of *S. marcescens* cells to polystyrene. Adhesion of *S. marcescens* cells to polystyrene was recorded in the presence of saliva that had undergone dialysis and reconstitution. To bacterial suspension (4 ml), 2 ml of PBS (column 1), untreated saliva (column 2), or dialyzed saliva (column 3) were added and, after 15 min incubation, adhesion (relative to column 1) to polystyrene wells was determined.

DISCUSSION

Cell surface hydrophobicity has been implicated as an important factor in mediating bacterial adhesion to various surfaces in the open environment, as well as within the host (Rosenberg & Kjelleberg, 1986; Marshall, 1984). The extent to which hydrophobic interactions mediate adhesion to oral surfaces (e.g. the tooth surface, saliva-coated hydroxyapatite and buccal epithelial cells) is currently unclear, but several lines of evidence suggest its importance: (i) most oral laboratory strains (Nesbitt *et al.*, 1982; Gibbons & Etherden, 1983; Olsson & Westergren, 1982), fresh isolates (Weiss *et al.*, 1982), and dispersed plaque microorganisms (Rosenberg *et al.*, 1983b) are hydrophobic, as determined by their ability to adhere to hydrocarbon droplets; (ii) correlations have been shown between bacterial hydrophobicity and adhesion to model tooth surfaces (Nesbitt *et al.*, 1982; Gibbons & Etherden, 1983; Olsson & Westergren, 1982), as well as *in situ* adhesion within the oral cavity (Svanberg *et al.*, 1984; Weiss *et al.*, 1985); (iii) amphipathic agents that interfere with bacterial attachment to

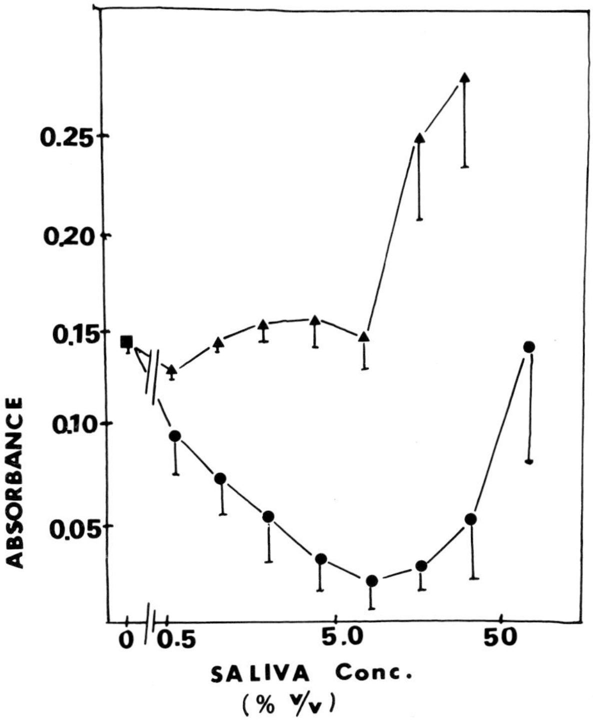

Fig. 4 Effect of unextracted and extracted saliva on adhesion of *A. calcoaceticus* RAG-1 to polystyrene. RAG-1 cells were tested for adhesion to polystyrene in the presence of various hexadecane pre-extracted (upper curve; ▲) and nonextracted (lower curve; ●) saliva concentrations.

hydrophobic surfaces similarly inhibit adhesion to oral epithelial cells and desorb normal flora (Rosenberg *et al.*, 1983a).

Hydrophobic interactions are particularly sensitive to the makeup of the bulk aqueous medium (Rosenberg & Kjelleberg, 1986). Thus, the amphipathic nature of saliva may play a major role in modulating oral adhesion (Rosenberg *et al.*, 1983c). The aim of the present investigation was to study the effect of saliva on adhesion of two hydrophobic bacterial strains to a hydrophobic surface (polystyrene) in a non-oral system, in which stereospecific interactions would be minimized.

Polystyrene is a convenient hydrophobic test surface that has been used in various adhesion studies (Fletcher, 1976; Rosenberg, 1981; Klotz *et al.*, 1985). Several investigators have recently employed this substratum to study the adhesion properties of oral strains. For example, the lack of ability of *Streptococcus sanguis* mutant to adhere to polystyrene correlated with its inability to adhere to saliva-coated hydroxyapatite (Gibbons *et al.*, 1983). Similarly, adhesion of *S. mutans* to microtiter wells has been employed to study complementation of adhesion-deficient mutants (Larrimore *et al.*, 1983).

The data demonstrate that component(s) present in saliva inhibit adhesion of *S. marcescens* RZ to polystyrene in a concentration-dependent fashion. This inhibition is probably due to the coating of the polystyrene surface rather than the cells themselves. Reduction of bacterial adhesion to polystyrene after "conditioning" of the plastic by various proteins was demonstrated by Fletcher (1976).

Hexadecane extraction of saliva (diluted 1:1 with PBS) brings about a complete loss in its ability to inhibit adhesion of *S. marcescens* to polystyrene, concomitant with a 24% decrease in protein content and an increase of 10·3 dynes·cm^{-1} in surface tension. These results suggest that the extraction procedure adsorbs amphipathic proteins, and perhaps other components from the bulk aqueous phase. Rosenberg *et al.* (1986) have shown that hexadecane extraction is a relatively mild method of extracting amphipathic biopolymers. The salivary constituents mediating the inhibition observed here are apparently able to interact with both hexadecane droplets and the polystyrene surface. Further experiments are under way to determine whether low molecular weight proteins, such as lysozyme, or other salivary constituents play a role in the observed inhibition.

Of interest are the results obtained with the non-oral, oil-degrading bacterium, *A. calcoaceticus* RAG-1. At low salivary concentrations, adhesion was inhibited, similar to that demonstrated for *S. marcescens*. However, high salivary concentrations actually promoted adhesion to the polystyrene surface. Promotion of adhesion was concomitant with saliva-mediated aggregation of RAG-1 cells. Although saliva-mediated aggregation of various oral microorganisms is common (Kashket & Donaldson, 1972), we are unaware of reports on saliva-mediated aggregation of species that are clearly not constituents of the normal oral microbiota, such as *A. calcoaceticus*. Preliminary data (not shown) suggest that (i) saliva-mediated aggregation of RAG-1 is common to samples obtained from most individuals; and (ii) the aggregation factor is probably directed against the thin fimbriae (Rosenberg *et al.*, 1982) of RAG-1 cells, as fimbriae-deficient mutant MR-481 is not similarly aggregated.

Although the factors responsible for the inhibition of adhesion of RAG-1 cells to polystyrene can be extracted from saliva by hexadecane as with *S. marcescens*, the component(s) mediating RAG-1 aggregation and concomitant increase in adherence are not extractable by this method. Thus, the increased adhesion of aggregated RAG-1 cells to the hydrophobic test surface is probably not due to hydrophobicity of the aggregating factor *per se*. The results demonstrate that saliva is capable of simultaneously inhibiting and promoting adhesion of a specific strain through separate and distinct components. The final result appears to be the net effect of these two mechanisms.

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References

Fletcher M (1976) The effects of proteins on bacterial attachment to polystyrene. *J Gen Microbiol* **94:** 400–404

Gibbons R J, Etherden I (1983) Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. *Infect Immun* **41:** 1190–1196

Gibbons R J, Etherden I, Skobe Z (1983) Association of fimbriae with the hydrophobicity of *Streptococcus sanguis* FC-1 and adherence to salivary pellicles. *Infect Immun* **41:** 414–417

Kashket S, Donaldson C G (1972) Saliva-induced aggregation of oral streptococci. *J Bacteriol* **112:** 1127–1133

Klotz S A, Drutz D J, Zajic J E (1985) Factors governing adherence of *Candida* species to plastic surfaces. *Infect Immun* **50:** 97–101

Larrimore S, Murchison H, Shiota T, Michalek S, Curtis III R (1983) *In vitro* and *in vivo* complementation of *Streptococcus mutans* mutants defective in adherence. *Infect Immun* **42:** 558–566

Lowry O H, Rosebrough N J, Farr L A, Randall R J (1951) Protein measurements with the Folin phenol reagent. *J Biol Chem* **193:** 265–275

Marshall K C (ed) (1984) *Microbial Adhesion and Aggregation.* Springer, Berlin Heidelberg New York Tokyo

Nesbitt W E, Doyle R J, Taylor K G (1982) Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxyapatite. *Infect Immun* **38:** 637–644

Olsson J, Westergren G (1982) Hydrophobic properties of oral streptococci. *FEMS Microbiol Lett* **15:** 319–323

Rosenberg M (1981) Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Appl Environ Microbiol* **42:** 375–377

Rosenberg M (1984) Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. *J Bacteriol* **160:** 480–482

Rosenberg M, Kjelleberg S (1986) Hydrophobic interactions: role in bacterial adhesion. In: Marshall K C (ed) *Advances in Microbial Ecology.* Plenum Publishing Corp., New York, pp. 353–393

Rosenberg M, Bayer E A, Delarea J, Rosenberg E (1982) Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *Appl Environ Microbiol* **44:** 929–937

Rosenberg E, Gottlieb A, Rosenberg M (1983a) Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. *Infect Immun* **39:** 1024–1028

Rosenberg M, Judes H, Weiss E (1983b) Cell surface hydrophobicity of dental plaque microorganisms *in situ. Infect Immun* **42:** 831–834

Rosenberg M, Rosenberg E, Judes H, Weiss E (1983c) Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. *FEMS Microbiol Lett* **20:** 1–5

Rosenberg M, Zosim Z, Regimov N, Eli I (1986) Separation of amphipathic proteins based on adsorption to hexadecane:water interfaces. *Prep Biochem* **16:** 133–141

Svanberg M, Westergren G, Olsson J (1984) Oral implantation in humans of *Streptococcus mutans* strains with different degrees of hydrophobicity. *Infect Immun* **19:** 497–504

Weiss E, Rosenberg M, Judes H, Rosenberg E (1982) Cell surface hydrophobicity of adherent oral bacteria. *Current Microbiol* **7:** 125–128

Weiss E, Judes H, Rosenberg M (1985) Adherence of a non-oral hydrophobic bacterium to the human tooth surface. *Dental Medicine* **3:** 11–13

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A simple method for estimating oral microbial levels

Mel Rosenberg[1, 2], Mira Barki[1] and Susana Portnoy[1]

[1]*Maurice and Gabriela Goldschleger School of Dental Medicine;* [2]*Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel*

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Summary

A simple test is described which enables estimation of oral microbial levels. The test is based on three steps: (1) oral rinsing with ultrahigh-temperature-sterilized milk, (2) expectoration of the sample into a vessel containing methylene blue, and (3) measurement of the time required for colour change at the bottom of the vessel, indicating anaerobiosis. The results correlate highly with microbial counts (colony-forming units, aerobic conditions) in the expectorate. The method may have potential clinical applications as a test for measurement of oral hygiene levels.

Key words: Method; Methylene blue; Milk; O_2; Oral microorganism

Introduction

Various oral ailments are due to microbial activity in the mouth. In some cases, elevated levels of a single oral species (or closely related species), can be correlated with diseased states. For example, dental caries is usually associated with *Streptococcus mutans* levels [1]. On the other hand, periodontal disease is accompanied by elevated levels of at least 57 bacterial species [2], many of them anaerobic or microaerophilic. Halitosis (bad breath) is similarly due to a variety of microorganisms which produce hydrogen sulphide, mercaptans, and other cytotoxic putrid volatile gases, generally under anaerobic conditions [3].

It is likely that microbial O_2 depletion in various oral niches is a prerequisite for periodontal disease development, halitosis, and perhaps additional oral ailments. Thus, oral levels of aerobic microorganisms may have considerable clinical importance. In the present report, we describe a simple test for estimating oral microbial levels, based on the rate of O_2 consumption in expectorated samples.

Correspondence to: M. Rosenberg, Maurice and Gabriela Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel.

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Materials and Methods

The test is based on the preliminary observation that sterile milk, following rinsing in the oral cavity, appears to be quickly fermented. This phenomenon was most pronounced upon awakening when oral microbial counts are high. Addition of methylene blue (a commonly used indicator for microbial levels in milk) to the expectorate revealed a blue-to-white colour change (indicative of decreasing redox potential caused by O_2 consumption) within as little as 5 min.

Test procedure

To test whether the rate of colour change can be correlated with microbial levels in the expectorates, the following protocol was devised: (1) volunteers rinsed their mouths vigorously with 10 ml sterile ultrahigh-temperature 3% milk (Tnuva, Rehovot, Israel, pH 6.5), for 30 s; (2) the expectorate was collected into a small cup; (3) 3 ml expectorate were immediately removed into a sterile disposable test tube (Cel-Cult, Sterilin, Feltham, U.K.; 10 mm inner diameter, 7.5 cm length) containing 0.12 ml 0.1%

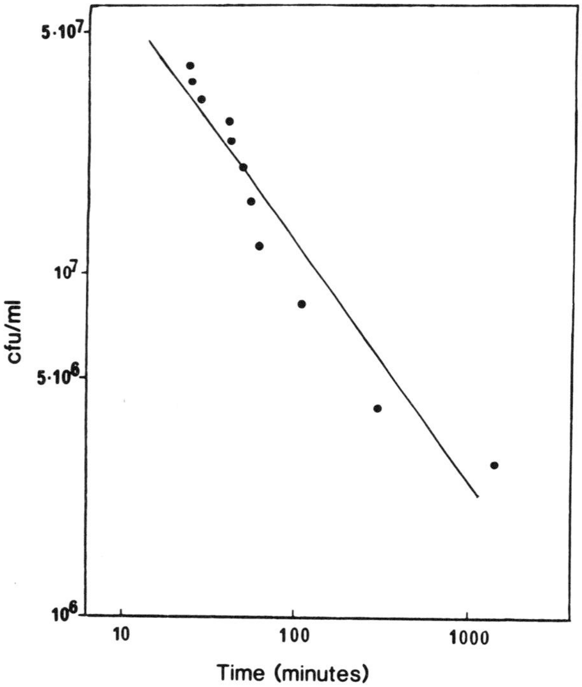

Fig. 1. Effect of expectorate dilution on time required for colour change. One of us (M. Rosenberg) rinsed his mouth vigorously for 30 s with 10 ml ultrahigh-temperature-sterilized milk. Expectorate was serially diluted with sterile milk prewarmed to 37 °C. Diluted samples (3 ml) were added to test tubes containing 0.12 ml 0.1% methylene blue. For microbial counts, serial 10-fold dilutions of original sample in sterilized milk were plated onto brain-heart infusion agar and incubated aerobically at 37 °C. Results are expressed as cfu$\cdot$ml^{-1} as a function of time required for colour change at bottom of test tube.

aqueous solution of methylene blue; (4) the test tube was capped, mixed briefly, and allowed to stand at room temperature (21 – 23 °C) over a mirror; (5) the time required for a colour change (blue-to-white) within a 6 mm diameter circle (drawn onto the bottom of the test tube) was recorded.

Microbial counts

For microbial counts, each sample was serially diluted using sterilized milk. 10-μl aliquots were plated onto brain-heart infusion agar (Difco) and colonies enumerated following overnight incubation at 37 °C.

Results and Discussion

The test correlates highly with microbial levels in the expectorate samples, as determined by colony-forming units (cfu) following incubation under aerobic conditions. Fig. 1 illustrates the correlation between the time required for the colour change and

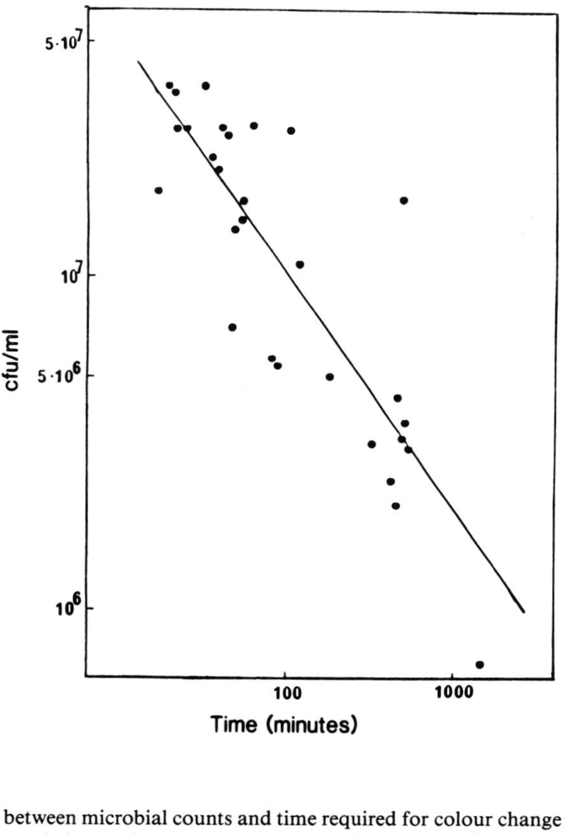

Fig. 2. Correlation between microbial counts and time required for colour change in 29 adult volunteers. They were asked to rinse their mouths vigorously for 30 s with 10 ml sterilized milk. 3 ml expectorate were added to test tubes containing methylene blue, as described in legend to Fig. 1. Appropriate dilutions of each sample were plated for microbial counts as described in Materials and Methods. Results are expressed as cfu·ml^{-1} as a function of time required for colour change.

the microbial level in serial dilutions of a single expectorate. The plot of the logarithm of cfu as a function of the logarithm of the time required for the colour change yields a straight line (r = -0.959, $P<0.001$), with a slope of -0.687. An experiment in which 29 volunteers (students and staff of the Dental School) participated, is shown in Fig. 2. Again, an excellent correlation was observed (r = -0.843, $P<0.001$), with a slope almost identical to that of Fig. 1 (-0.698). Times required for colour change among the volunteers ranged from 16 to 1440 min, with a median of 62 min. According to Fig. 2, each doubling in the time required for the colour change corresponds to a 38% decrease in the level of expectorated microorganisms.

Microbial examination of expectorates revealed a variety of microorganisms which were able to reduce milk samples in pure cultures. Traudt and Kleinberg [4] have shown that most microorganisms in supragingival dental plaque readily consume O_2. Offenbacher and coworkers [5] have recently shown that periodontal (subgingival) plaque microorganisms often prefer or require a preexisting bacterial community for colonization and proliferation; it is reasonable to assume that such a relationship exists between strictly anaerobic periodontal 'pathogens' and closely associated aerobic microbiota.

Initial data show highly significant correlations between results of this test and clinical parameters, including plaque index and gingivitis in adults (typescript in preparation). The simplicity of the test and the rapidity with which positive results (i.e., high microbial levels) are obtained, suggest its potential as a clinical and research tool. Further development of the technique into a commercial kit is presently underway.

References

1 Kristoffersson, K., Grondahl, H.-G. and Bratthall, G. (1985) The more *Streptococcus mutans*, the more caries on approximal surfaces. J. Dent. Res. 64, 58–61.

2 Moore, W. E. C., Holdeman, L. V., Cato, E. P., Smibert, R. M., Burmeister, J. A., Palcanis, K. G. and Ranney, R. R. (1985) Comparative bacteriology of juvenile periodontitis. Infect. Immun. 48, 507–519.

3 Tonzetich, J. (1977) Production and origin of oral malodor: a review of mechanisms and methods of analysis. J. Periodontol. 48, 13–20.

4 Traudt, M. and Kleinberg, L. (1988) Bacteria in human dental plaque responsible for its oxygen uptake activity. J. Dent. Res. 67, 204.

5 Offenbacher, S., Costopoulos, S. V., Odle, B. M. and Van Dyke, T. E. (1988) Microbial colonization patterns of loosely adherent subgingival plaque in adult periodontitis. J. Clin. Periodontol. 15, 53–59.

6 Rosenberg, M. and Gabbay, J. (1987) Halitosis – a call for affirmative action. Dent. Med. 5, 13–15.

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Adhesion of Non-coccal Dental Plaque Microorganisms to Buccal Epithelial Cells: Inhibition by Saliva and Amphipathic Agents

MEL ROSENBERG*†‡, MEIR TAL†, ERVIN WEISS† and SERGIO GUENDELMAN†

The Maurice and Gabriela Goldschleger School of Dental Medicine†, and Department of Human Microbiology, Sackler Faculty of Medicine‡, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel.

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In the present investigation, the *in vitro* adhesion of dispersed non-coccal dental plaque bacteria to buccal epithelial cells was examined. Human buccal epithelial cells, incubated in the presence of dispersed supragingival dental plaque, bound averages of ca. 6–15 non-coccal forms/epithelial cell, as opposed to indigenous levels of only 0·2–0·6 on buccal epithelial cells incubated in buffer alone. Adhesion was strongly inhibited by amphipathic agents (Tween 80, sodium dodecyl sulphate, and emulsan), and by saliva. The data suggests firstly that non-coccal bacteria can adhere to epithelial cells *in vitro*, but may be prevented from doing so *in situ* by saliva, and secondly that hydrophobic interactions may help mediate the observed adhesion.

KEY WORDS—Adhesion; Dental plaque; Epithelial cells; Saliva; Amphipathic agents.

INTRODUCTION

The various surfaces within the human mouth are known to harbour widely differing microbial populations. For example, high levels of *Streptococcus salivarius* are found attached to the tongue dorsum and buccal mucosa, but low levels are observed adhering to tooth surfaces. *Streptococcus mutans*, on the other hand, colonises tooth surfaces but not soft oral tissues.[3] Striking differences in microbiota are also observed microscopically: whereas coccal forms are predominant on the buccal epithelium, non-coccal types, such as rods, filamentous, and fusiform bacteria constitute a large proportion of dental plaque.[7] It has been proposed that such differences reflect specific adhesion mechanisms whereby oral bacteria adhere exclusively to those surfaces on which they are able to proliferate.[3,4] An alternate explanation is that oral bacteria may adhere through non-specific hydrophobic interactions to various saliva-bathed surfaces, including those on which they do not proliferate.[11–14]

In this investigation, we addressed the following question: do non-coccal bacteria from dental plaque adhere appreciably to buccal epithelial cells *in vitro*, despite the very low numbers observed *in vivo*? To this end, both dental plaque bacteria and buccal epithelial cells were scraped from the human mouth and tested directly to avoid alterations in adhesion properties as a result of *in vitro* transfers. The results indicate firstly that non-coccal dental plaque microbiota do adhere to buccal epithelial cells *in vitro*, and secondly that this adhesion is blocked by saliva and amphipathic agents.

MATERIALS AND METHODS

Plaque microorganisms

Supragingival dental plaque (several days old) was scraped with a sterile curette from the mouths of several healthy adult volunteers,[12] and immediately suspended in phosphate-buffered saline (PBS; 8·0 g NaCl, 0·2 g KCl, 1·15 $NaHPO_4$, 0·2 g KH_2PO_4, and distilled water to 1 l, pH 7·2). The plaque was then washed twice by centrifugation and suspended in PBS. Plaque was dispersed by shearing[10] for four 15 s intervals using an Ultra-Turrax (Janke and Kunkel Ikawerk, Staufe, FRG). Clumps of non-dispersed plaque microorganisms were removed by centrifugation for 1 min at 30 g. Microscopic observation of the supernatant revealed that it was mainly composed of single cells; rods, filaments, fusiforms and coccal forms were prevalent; the

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*Author to whom correspondence should be addressed.

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proportion of coccal and non-coccal forms was approximately 1:1. The dispersed plaque suspension was adjusted to an optical density of ca. 1·4 at 400 nm (ca. 10^9 cells/ml) using a Gilford 240 spectrophotometer, 1 cm light path.

Emulsan is an amphipathic bacterial polysaccharide which has been previously shown to inhibit bacterial adhesion.[8] Emulsan was prepared from *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) as previously described.[8]

Whole, unstimulated saliva, was collected from one of the authors (S.G.), clarified by centrifugation (ca. 10 000 g for 5 min), diluted in PBS as indicated, and used immediately.

Epithelial cells were obtained as previously described[8,10] by scraping the buccal mucosa of healthy volunteers with wooden sticks. The pooled epithelial cells were washed twice and suspended in PBS to a concentration of ca. 10^6 cells/ml.

An epithelial cell suspension (0·5 ml) was mixed with 0·1 ml of dispersed plaque microorganisms, and 0·066 ml of PBS containing appropriate dilutions of Tween 80, SDS, emulsan or saliva. Mixtures were rotated end-over-end for 60 min at room temperature. Epithelial cells were separated from non-adherent plaque by filtration through 12 μm diameter pore size filters (Nuclepore Corp., Pleasanton, Calif.) The filters were washed three times with 20 ml PBS. Epithelial cells and attached bacteria were stained differentially using Hemacolor rapid stain (Merck and Co., Inc., Rahway, N.J.).[8,10] The number of bacteria which were clearly non-coccal forms adhering to 100 individual epithelial cells was scored microscopically, at a magnification of 1000; each experiment was repeated at least twice.

The t-test was used to test for significance.

RESULTS

Initially, we tested the ability of washed and dispersed plaque microorganisms to adhere to buccal epithelial cells collected separately from three volunteers. The results are presented in Table 1. In all cases, buccal epithelial cells incubated in the presence of dispersed plaque bound non-coccal bacteria in high numbers as opposed to extremely low levels adhering to buccal epithelial cells incubated in the presence of buffer alone. Despite the variations commonly observed in bacterial adhesion to freshly isolated buccal epithelial cells,[1] adhesion was in each case highly significant ($p < 0{\cdot}0005$), compared to the buffer control. Overall, epithelial cells incubated in the presence of plaque bound an average of 8·8 non-coccal microorganisms/epithelial cell as opposed to 0·30 indigenous non-coccal microorganisms per cell present on epithelial cells incubated in the presence of buffer alone. Adhesion of dispersed plaque microorganisms to a buccal epithelial cell is shown in Figure 1, compared to a cell incubated in the presence of buffer alone. Non-coccal forms appeared to adhere directly to the epithelial cells, and occasionally along the outer border of the epithelial cells. In order to study the effects of various agents on adhesion, subsequent experiments were performed using pooled samples of both buccal epithelial cells and dental plaque.

Table 1. Adhesion of dispersed plaque microorganisms obtained from three volunteers to human buccal epithelial cells

Volunteer number	Adhesion to epithelial cells* Dispersed plaque	Control (buffer)	Different from control ($p<$)
1	6·5±8·2	0·06±0·24	0·0005
2	9·5±16·5	0·21±0·52	0·0005
3	10·5±9·3	0·64±1·8	0·0005

*Buccal epithelial cells were incubated together with dispersed dental plaque microorganisms, or alternatively in buffer alone, as described in Materials and Methods. Results are presented as number of adherent non-coccal forms per epithelial cell ± standard deviation.

Emulsan inhibits bacterial adhesion to hydrocarbon and epithelial cells in a concentration-dependent manner.[8] A typical experiment showing the effect of emulsan on adhesion is shown in Figure 2. Emulsan concentrations above 2 μg/ml were highly inhibitory; for example, at 5 μg/ml emulsan, only 0·6 non-coccal bacterium/epithelial cell were observed, as opposed to 14·8 non-coccal forms/epithelial cell, in the absence of emulsan. A slight, reproducible decrease in inhibition ability was observed for higher emulsan concentrations, probably due to increased viscosity.

Tween 80 and sodium dodecyl sulphate at final concentrations of 200 μg/ml, inhibited adhesion by 80 per cent and 90 per cent, respectively (Table 2), compared with the surfactant-free control. In both cases the inhibition was highly significant ($p < 0{\cdot}0005$) as compared to adhesion in the absence of added surfactant.

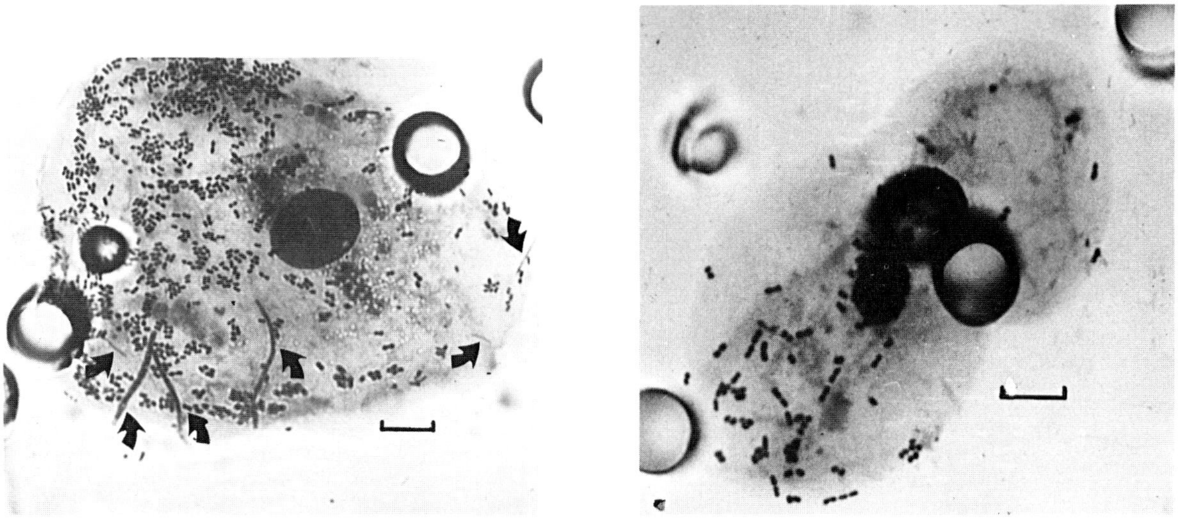

Figure 1. Adhesion of dental plaque microorganisms to buccal epithelial cells. Buccal epithelial cells were incubated in the presence or absence of dispersed dental plaque as described in Materials and Methods. Figure 1A—Adhesion of non-coccal microorganisms to epithelial cell can be readily observed (arrows); Figure 1B—Only cocci are observed on epithelial cells incubated in the presence of buffer alone. Bars represent 10 μ. Dark circles are nuclei; light circles are pores in the filter

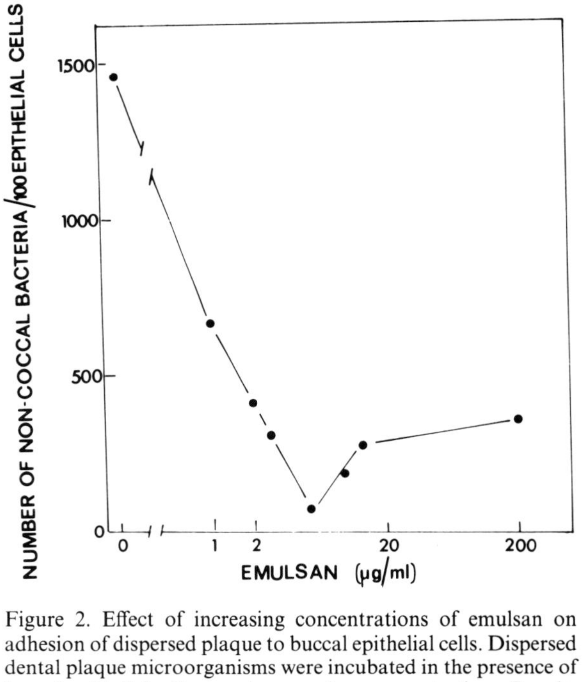

Figure 2. Effect of increasing concentrations of emulsan on adhesion of dispersed plaque to buccal epithelial cells. Dispersed dental plaque microorganisms were incubated in the presence of buccal epithelial cells at various emulsan concentrations. Results are expressed as the number of non-coccal bacteria adhering to 100 epithelial cells, as a function of emulsan concentration

Whole, human saliva inhibited adhesion in a concentration-dependent manner. Results of a typical experiment are shown in Figure 3. Saliva concentrations as low as one per cent (v/v) almost completely blocked adhesion.

DISCUSSION

Under the conditions used, non-coccal microorganisms directly obtained from supergingival dental plaque adhere *in vitro* to human buccal epithelial cells. Adhesion levels of ca. 6–15 non-coccal bacteria/per epithelial cell were observed, as opposed to indigenous levels of ca. 0·3. These data indicate that although non-coccal dental plaque microorganisms adhere poorly to buccal epithelial cells *in vivo*, despite the abundance of such forms in dental plaque and their proximity to the buccal epithelium, they readily do so in an *in vitro* system. The simplest explanation for this observation is that such microorganisms possess the ability to adhere to buccal epithelial cells, but are prevented from doing so *in vivo* by saliva. Saliva (1 per cent, v/v) similarly inhibits adhesion of coccal plaque microbiota to buccal epithelial cells (S. Guendelman, unpublished data). Moreover, saliva has been shown to inhibit bacterial adhesion to a solid surface (polystyrene) in a concentration dependent manner.[2]

Non-coccal types which do manage to overcome the saliva barrier and adhere *in vivo* to buccal epithelial cells may be unable to grow on them, thus reducing their proportion in relation to the indigenous coccal types which proliferate on the buccal epithelium.

Table 2. Effect of surfactants on the adhesion of non-coccal dental plaque microorganisms to buccal epithelial cells*

	Adhesion following incubation in				
Additions†	dispersed plaque	buffer	Net adhesion‡	Percent inhibition§	Different from control ($p<$)
None	14·92	0·15	14·77	—	
Tween-80	3·18	0·23	2·95	80	0·0005
SDS	1·73	0·18	1·55	90	0·0005

*Buccal epithelial cells were incubated together with dispersed dental plaque microorganisms, or alternatively in buffer alone, in the presence or absence of surfactants. Results are presented as number of adherent non-coccal forms per epithelial cell.

†Surfactants were added to a final concentration of 0·02 per cent prior to incubation.

‡The number of non-coccal forms adhering per epithelial cell following incubation in the presence of dispersed plaque less the number adhering following incubation in the presence of buffer alone.

§With respect to net adhesion of control vs. experimentals.

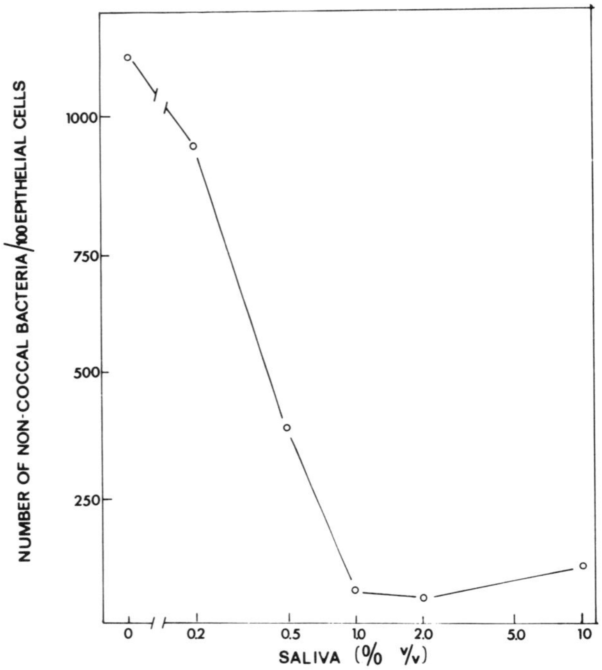

Figure 3. Effect of increasing concentrations of whole saliva on adhesion of dispersed plaque to buccal epithelial cells. Dispersed dental plaque microorganisms were incubated in the presence of buccal epithelial cells at various saliva concentrations. Results are expressed as the number of non-coccal bacteria adhering to 100 epithelial cells, as a function of final saliva concentration (v/v)

The observed inhibition of adhesion by amphipathic agents raises the possibility that hydrophobic interactions are involved. Hydrophobic interactions have been implicated in a wide range of adhesion phenomena, in the host and in the open environment.[9] Recent reports have suggested that hydrophobic interactions help promote bacterial adhesion to buccal epithelial cells, and to the tooth surface itself.[3,5-15] For example, the hydrophobic non-oral bacterium *A. calcoaceticus* RAG-1 adheres both to buccal epithelial cells and to the tooth surface whereas its non-hydrophobic mutant does not.[10,13] Although it cannot be completely excluded that sodium dodecyl sulphate and Tween 80 may have inhibited adhesion by denaturing or otherwise altering cell surface components, emulsan is an amphipathic polysaccharide which has been shown to inhibit hydrophobic interactions at low concentrations without damaging cells.[8,9] The potent inhibition of saliva may also be due to the presence of amphipathic components which inhibit hydrophobic interactions.[2] Thus, the results obtained in the present study are consistent with the premise that bacterial hydrophobicity contributes to oral adhesion.[10] It should, however, be emphasised that participation of stereospecific interactions cannot be excluded; firstly tightly-bound clumps of dental plaque bacteria which may adhere via other mechanisms were not dispersed by the shearing procedure and thus not included in the experiments; secondly, the bacteria which adhered even in the presence of amphipathic agents may have done so via alternative mechanisms; and lastly, hydrophobic and stereospecific interactions may act in concert to promote oral adhesion.[6]

In a previous study, the ability of dispersed plaque microorganisms, obtained directly from the mouth, to adhere to various hydrocarbon droplets was demonstrated.[12] The present study extends this finding to include adhesion to buccal epithelial cells. The use of dispersed plaque microorganisms to study adhesion has two main advantages; the outer microbial cell surfaces are not altered by *in vitro* growth conditions and culture transfer,[15] and the general proportions of the various kinds of microorganisms present in the sample are maintained.[5,12,14]

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REFERENCES

1. Abraham SN, Beachey EH, Simpson WA. (1983). Adherence of *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* to fibronectin-coated and uncoated epithelial cells. *Infection and Immunity* **41,** 1261–1268.
2. Eli I, Judes H, Rosenberg M. (1989). Saliva-mediated inhibition and promotion of bacterial adhesion to polystyrene. *Biofouling* (in press).
3. Gibbons RJ. (1980). Adhesion of bacteria to the surfaces of the mouth. In: Berkeley RCW, Lynch JM, Melling J, Rutter PR, Vincent B (eds). *Adsorption of Microorganisms to Surfaces*. Ellis Horwood, Chichester, England, pp. 283–288.
4. Gibbons RJ. (1984). Adherent interactions which may affect microbial ecology in the mouth. *Journal of Dental Research* **63,** 378–385.
5. Leach SA, Agalamanyi. (1984). Hydrophobic interactions that may be involved in the formation of dental plaque. In: Ten Cate JM, Leach SA, Arends J (ed) *Bacterial Adhesion and Preventive Dentistry*. IRL Press, Oxford, p 43–50.
6. Nesbitt WE, Doyle RJ, Taylor KG. (1982). Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite. *Infection and Immunity* **38,** 637–644.
7. Nolte WA. (1981) Oral microflora. In: Nolte WA (ed) *Oral Microbiology*. CV Mosby Co., St Louis, pp. 193–228.
8. Rosenberg E, Gottlieb A, Rosenberg M. (1983). Inhibition of bacterial adherence to epithelial cells and hydrocarbons by emulsan. *Infection and Immunity* **39,** 1024–1028.
9. Rosenberg M, Kjelleberg S. (1986). Hydrophobic interactions: role in bacterial adhesion. *Advances in Microbial Ecology* **9,** 353–393.
10. Rosenberg M, Perry A, Bayer EA, Gutnick DL, Rosenberg E, Ofek I. (1981). Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. *Infection and Immunity* **33,** 29–33.
11. Rosenberg M, Rosenberg E, Judes H, Weiss E. (1983). Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. *FEMS Microbiology Letters* **20,** 1–5.
12. Rosenberg M, Judes H, Weiss E. (1983). Cell surface hydrophobicity of dental plaque microorganisms *in situ*. *Infection and Immunity* **42,** 831–834.
13. Weiss E, Judes H, Rosenberg M. (1985). Adherence of a non-oral hydrophobic bacterium to the human tooth surface. *Dental Medicine* **3,** 11–13.

Chapter 23

Microbial Films in the Mouth: Some Ecologically Relevant Observations

Mel Rosenberg

INTRODUCTION

One need not travel great distances to investigate an exciting, naturally occurring macroscopic biofilm—a look in the mirror is quite sufficient. Dental plaque, the macroscopic accumulation of bacteria on tooth surfaces, is a convenient, close-at-hand biofilm of great importance to humans. Dental caries and periodontal disease, the two most widespread diseases of modern humans, are linked to the activity of microorganisms on tooth surfaces (7, 12, 17). The soft tissues in the mouth are also colonized by biofilms, which are generally sparser than dental plaque, presumably owing to the shedding (desquamation) of the outermost epithelial cell layers. Finally, dental restorations, dentures, and devices are similarly prone to rapid biofouling in the oral cavity.

Microbial accumulations in the oral cavity appear to share several characteristics with biofilms in the open environment, as follows. (i) Similar to microbial films which form on surfaces in contact with water flow (pipes, rocks and sediments, submerged aquatic life, etc.), oral microorganisms must adhere to remain in their ecological niche and to survive. (ii) Similar to many environmental biofilms, bacterial accumulation on oral surfaces entails a complex physical and physiological coexistence involving a wide variety of bacterial species (7, 12). (iii) Maturing dental plaque results in a succession of anaerobic and acid-tolerant populations in close proximity to the tooth surface (7, 12). (iv) With prolonged passage of time, dental plaque accumulations become increasingly recalcitrant and calcified (dental calculus) and may be removed only by strong mechanical action (e.g., sonic oscillation). (v) Analogous to the chemical and structural changes in the underlying substratum as a result of many biofilms (e.g., corrosion of metals), tooth decay (dental caries) results from destruction of the protective enamel layer, primarily by acid accumulation (7, 12, 17). Finally, similar to the odorous, volatile gases produced by microbial mats and other biofilms, volatile gas elaborated by microbial accumulations in the mouth itself is the primary cause of bad breath (halitosis) (33, 36).

In this chapter I discuss some "ecological" aspects of oral biofilms which have attracted the interest of investigators in this

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Mel Rosenberg • The Maurice and Gabriela Goldschleger School of Dental Medicine and the Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel.

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and other laboratories: (i) adhesion, colonization, and desorption of oral bacteria; (ii) sampling and localization of adherent microbial populations on tooth surfaces; and (iii) measurement and monitoring of volatile, sulfur-containing gases in the oral cavity.

ADHESION, COLONIZATION, AND DESORPTION

Adhesion and Colonization

Cleaned surfaces inserted into the oral cavity or, alternatively, immersed in saliva become conditioned with a coat of salivary components prior to microbial adhesion (7, 12). This layer, termed the acquired pellicle, may differ to some extent in composition, distribution, or juxtaposition of adsorbed salivary molecules, depending on the substratum characteristics; it is likely, however, that many salivary components adsorb to a wide variety of oral surfaces (12).

Researchers studying the oral cavity are in general agreement that microbial adhesion is a crucial initial process in the colonization of oral surfaces; however, the actual mechanisms which promote oral adhesion have been a subject of debate for years (7, 12, 15–17, 30, 31). Similarly, the subsequent role of adhesion in biofilm buildup on oral surfaces is unclear: dental plaque accumulation appears to be due primarily to outgrowth of already attached bacteria, rather than to adsorption of free cells from saliva (12).

One striking observation of oral biofilms is the heterogeneity of their microbial composition. For example, high levels of *Streptococcus salivarius* are found attached to the tongue dorsum and buccal surfaces, but very low levels are observed in dental plaque. *Streptococcus mutans*, on the other hand, colonizes tooth surfaces but not soft oral tissues and is thus absent from mouths that are free of teeth or artificial hard surfaces (7). Striking differences in microbiota are also observed microscopically: whereas coccal forms are predominant on buccal epithelial cells, noncoccal forms, such as rods, filamentous bacteria, and fusiform bacteria, constitute a large proportion of dental plaque (7). Gibbons et al. have postulated that these differences reflect specific adhesion mechanisms (7, 10). Thus, according to this hypothesis, oral bacteria adhere with high affinity to surfaces on which they are able to proliferate, but adhere poorly to surfaces which they do not colonize.

During recent years, there has been an increasing number of reports that hydrophobic interactions play a significant role in bacterial adhesion in the open environment. In 1982, Weiss et al. (40) and Nesbitt et al. (21) reported findings suggesting that bacterial hydrophobicity may be important for oral adhesion as well. During the past 5 years, this possibility has been explored extensively in several laboratories.

The following lines of evidence support a major role for bacterial hydrophobicity in oral adhesion. (i) Many oral bacteria exhibit pronounced cell surface hydrophobicity, as measured by adhesion to hydrocarbons; they include fresh oral isolates (40), caries-associated and periodontal disease-associated laboratory strains (5, 8, 14, 19, 21, 23, 42), dental plaque itself (29), and bacteria obtained directly from saliva (15, 16). (ii) In several cases, clear-cut correlations were observed between adhesion to saliva-coated hydroxyapatite (SHA) beads (an in vitro tooth surface model system) and cell surface hydrophobicity in various oral strains (8, 19, 23) and following various treatments (11, 22); moreover, adhesion to SHA can be blocked by hydrophobic-bond-breaking agents, e.g., thiocyanate and tetramethyl urea (21, 38). (iii) Nonhydrophobic mutants of hydrophobic strains are deficient in adhering to tooth surfaces (39) or SHA (6, 9, 19). (iv) In an in vivo study, Svanberg et al. showed that a hydrophobic fresh isolate of *S. mutans* is retained in the human oral cavity, whereas its nonhydrophobic variant is not

(34). (v) Emulsan, a bacterial amphiphile which blocks adhesion to hydrophobic surfaces, similarly inhibits the adhesion of oral and nonoral strains to buccal epithelial cells (26).

If hydrophobic interactions constitute a major mechanism for oral adhesion, one would expect that adhesion to various oral surfaces would not be as specific as previously suggested (31). Indeed, although filamentous plaque bacteria do not adhere significantly to buccal epithelial cells in situ, adhesion readily takes place in a saliva-free in vitro system (35). Moreover, this binding is inhibited by amphipathic agents and saliva (M. Rosenberg and S. Guendelman, unpublished data). Thus, the heterogeneity of microbial populations on various tooth surfaces may not be a reflection of their adhesion capabilities, but rather their relative ability to colonize and proliferate on various oral substrata (31).

Several recent investigations have challenged the role of hydrophobicity in oral adhesion. Van der Mei et al. (37), using six different methods for studying hydrophobicity, were unable to demonstrate a clear correlation between hydrophobicity and adhesion to SHA. Similarly, Wyatt et al. (43) reported that although all strains of *Streptococcus sanguis* tested adhered to hexadecane, large differences in adhesion to SHA were observed. Knox et al. (13), using continuous-culture-grown cells, found no correlation between adhesion to hexadecane and to SHA. Clark et al. (2) found a strong correlation between the relative hydrophobicity of 42 oral *Actinomyces* strains and adhesion to SHA, but were unable to observe inhibition of adhesion of SHA by the surfactant Tween 80.

These apparent disagreements can be resolved only through further research and improved methodology. It is likely that SHA may be an interesting model system with which to study adhesion, but that it does not clearly mimic the tooth surface itself; moreover, this technique is used with various significant alterations in different laboratories. Similarly, improved bacterial hydrophobicity tests could be of great assistance (30). In any case, it is worth emphasizing that the current hydrophobicity tests do not measure the same characteristics (32) and that no single test is definitive in measuring these properties (4, 20). This point is well illustrated by the recent reports of Weerkamp et al. (38) and van der Mei et al. (37). These investigators did find overall correlations between the adhesion of wild-type *S. salivarius* and various mutants to hexadecane and SHA. One mutant (HB-7), however, was exceptional in that it adhered to hexadecane but not to SHA. The investigators inferred from this observation that adhesion to SHA cannot be accounted for by bacterial hydrophobicity. However, this mutant adheres poorly to the hydrophobic plastic polymethylmethacrylate (37). Opposite observations have been made with *Acinetobacter calcoaceticus* BD4, which adheres to polystyrene but not hexadecane (27, 30). Finally, it should be emphasized that adhesion characteristics, including cell surface hydrophobicity, may be lost upon subculturing (41).

To summarize, the extent to which hydrophobic interactions mediate adhesion within the oral cavity remains unresolved at present. Some investigators have proposed a major role for cell surface hydrophobicity in mediating oral adhesion (16, 23, 31, 38); others have urged fellow researchers to desist this line of study altogether (25); still others have proposed that hydrophobic interactions act in concert with additional mechanisms (3, 5, 21, 38). Indeed, the findings that the lactose-sensitive coaggregation of oral bacteria may have hydrophobic components (18) suggest that a precise line between stereochemically specific and nonspecific adhesion mechanisms may be hard to draw. Finally, bacteria may adhere by one mechanism to other bacteria, which are themselves bound by another mechanism to the tooth surface (1).

Bacterial Desorption from Oral Surfaces

Among the treatments to eliminate or reduce microbial buildup on surfaces, desorption of attached cells is a logical choice, since inhibition of the initial adhesion processes is often unfeasible. Desorption itself is a more complicated process than inhibition of adhesion, since a bacterium may adhere via a multitude of molecular interactions, which are difficult to break simultaneously; moreover, bacterial elaboration of extracellular polysaccharides may help cement already adherent cells.

The observation that a large proportion of oral bacteria adhere to oil droplets prompted us to propose swishing with an oil-water rinse as a technique of desorbing adsorbed bacteria from oral surfaces (E. Weiss, M. Rosenberg, and H. Judes, U.S. patent 4,525,342, June, 1985). In such a case, the actual adhesion mechanisms are irrelevant as long as the affinity of the cells for the oil droplets can overcome the adhesive forces. In addition to their ability to desorb oral bacteria, oils have been shown to be capable of desorbing bacteria from polystyrene (28) and fish skin (32). During our studies, the possibility arose that oil droplets could also bind amphipathic odor-causing molecules (Weiss et al., patent) and perhaps serve to reduce halitosis levels. This, in turn, prompted us to look for a simple means of studying halitosis, which is discussed later on.

SAMPLING OF TOOTH SURFACES FOR SPECIFIC ADHERENT MICROORGANISMS

Several convenient methods (Rodac plates, contact dip slides, adhesive tape, etc.) exist for sampling microorganisms from smooth surfaces. However, the localization and sampling of specific microorganisms adhering to rough surfaces, e.g., tooth surfaces, present a problem. Recent studies have shown that early dental caries formation is linked to the presence of *S. mutans* (17). The levels of desorbed *S. mutans* cells in saliva are currently used to assess dental caries levels and susceptibility.

We were interested in trying to develop a simple technique to pinpoint the growth of *S. mutans* on the tooth surfaces, rather than measure the levels of desorbed cells in saliva. To this end, we obtained impressions of the tooth surface by using a tacky, malleable material. Cells not adhering to the impression material were washed off in tap water. Liquid agar-containing media were cooled to 45°C and poured over the impressions, but growth was difficult to discern. To simplify the process, we decided to attempt to incubate the impression in a liquid medium. To confine outgrowth to the solid/liquid interface, the major carbon and energy source (sucrose) was impregnated in the impression material but was absent from the liquid medium. By incorporating selective, chromogenic components in the liquid outgrowth medium, specific growth of *S. mutans* on the impression surfaces was clearly observable (31a). This technique may have clinical and ecological implications in mapping the presence of this microorganism on tooth surfaces and, perhaps, in providing an indication of incipient caries formation. Minor modifications of the method may also facilitate sampling and concomitant localization of adherent populations on rough surfaces in the environment.

HALITOSIS

As stated above, the production of foul oral odors is analogous to putrid-gas elaboration by microbial mats and biofilms in the open environment. However, halitosis is the cause of substantial human discomfort; unfortunately, relatively little research has been directed toward this problem, which is said to affect the majority of adults in the Western world (36). About 85 to 90% of halitosis cases are reported to be oral in origin: the direct consequence of metabolic activity of bacterial accumulations on various oral surfaces (36). Volatile sulfur-containing gases such as hydrogen sulfide, methyl and ethyl mercaptan, and dimethyl sulfide are a major factor in oral malodor. Most halitosis cases can be alleviated by proper oral hygiene and dental techniques (36). We have recently suggested that monitoring the levels of halitosis may aid the dental practitioner in treating this common ailment. Previously, halitosis measurements have been made by gas chromatography (33, 36), which is too complicated and time-consuming for routine clinical testing, or by an organoleptic panel of volunteers, which is similarly unsuitable. We have recently tested a portable, simple-to-use industrial sulfide monitor, which can measure sulfide concentrations below 0.1 mg/liter; initial results suggest that such testing can provide relevant quantitative data (24). The use of such instruments for field measurement of volatile sulfur produced by microbial mats and other biofilms may also be of interest.

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LITERATURE CITED

1. **Ciardi, J. E., G. F. A. McCray, P. E. Kolenbrander, and A. Lau.** 1987. Cell-to-cell interaction of *Streptococcus sanguis* and *Propionibacterium acnes* on saliva-coated hydroxyapatite. *Infect. Immun.* 55:1441–1446.
2. **Clark, W. B., M. D. Lane, E. Beem, S. L. Bragg, and T. T. Wheeler.** 1985. Relative hydrophobicities of *Actinomyces viscosus* and *Actinomyces naeslundii* strains and their adsorption to saliva-treated hydroxyapatite. *Infect. Immun.* 47:730–736.
3. **Cowan, M. M., K. G. Taylor, and R. J. Doyle.** 1987. Energetics of the initial phase of adhesion of *Streptococcus sanguis* to hydroxyapatite. *J. Bacteriol.* 169:2995–3000.
4. **Dillon, J. K., J. A. Fuerst, A. C. Hayward, and G. H. G. Davis.** 1986. A comparison of five methods for assaying bacterial hydrophobicity. *J. Microbiol. Methods* 6:13–19.
5. **Doyle, R. J., W. E. Nesbitt, and K. G. Taylor.** 1982. On the mechanism of adherence of *Streptococcus sanguis* to hydroxylapatite. *FEMS Microbiol. Lett.* 15:1–5.
6. **Fives-Taylor, P., and D. W. Thompson.** 1985. Surface properties of *Streptococcus sanguis* FW213 mutants nonadherent to saliva-coated hydroxyapatite. *Infect. Immun.* 47:752–759.
7. **Gibbons, R. J.** 1980. Adhesion of bacteria to surfaces of the mouth, p. 351–388. *In* R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), *Microbial Adhesion to Surfaces*. Ellis Horwood Ltd., Chichester, England.
8. **Gibbons, R. J., and I. Etherden.** 1983. Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. *Infect. Immun.* 41:1190–1196.
9. **Gibbons, R. J., I. Etherden, and Z. Skobe.** 1983. Association of fimbriae with the hydrophobicity of *Streptococcus sanguis* FC-1 and adherence to salivary pellicles. *Infect. Immun.* 41:414–417.
10. **Gibbons, R. J., D. M. Spinell, and Z. Skobe.** 1975. Selective adherence as a determinant of the host tropisms of certain indigenous and pathogenic bacteria. *Infect. Immun.* 13:238–246.
11. **Hesketh, L. M., J. E. Wyatt, and P. S. Handley.** 1987. Effect of protease on cell surface structure, hydrophobicity and adhesion of tufted strains of *Streptococcus sanguis* biotypes I and II. *Microbios* 50:131–145.
12. **Kleinberg, I.** 1982. Dynamics of the oral ecosystem, p. 229–244. *In* W. A. Nolte (ed.), *Oral Microbiology*. C. V. Mosby Co., St. Louis.
13. **Knox, K. W., L. N. Hardy, L. J. Markevics, J. D. Evans, and A. J. Wicken.** 1985. Comparative studies on the effect of growth conditions on adhesion, hydrophobicity, and extracellular protein profile of *Streptococcus sanguis* G9B. *Infect. Immun.* 50:545–554.
14. **Kozlovsky, A., Z. Metzger, and I. Eli.** 1987. Cell surface hydrophobicity of *Actinobacillus actinomycetemcomitans* Y_4. *J. Clin. Periodontol.* 14:370–372.
15. **Leach, S. A.** 1980. A biophysical approach to interactions associated with the formation of the matrix of dental plaque, p. 159–183. *In* S. A. Leach (ed.), *Dental Plaque and Surface Interactions in the Oral Cavity*. IRL Press, Oxford.
16. **Leach, S. A., and E. A. Agalamanyi.** 1984. Hydrophobic interactions that may be involved in the formation of dental plaque, p. 43–50. *In* J. M. ten Cate, S. A. Leach, and J. Arends (ed.), *Bacterial*

Adhesion and Preventive Dentistry. IRL Press, Oxford.

17. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* 50:353–380.
18. **McIntire, F. C., L. K. Crosby, and A. E. Vatter.** 1982. Inhibitors of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34: β-galactosides, related sugars, and anionic amphipathic compounds. *Infect. Immun.* 36:371–378.
19. **Morris, E. J., N. Ganeshkumar, and B. C. McBride.** 1985. Cell surface components of *Streptococcus sanguis*: relationship to aggregation, adherence, and hydrophobicity. *J. Bacteriol.* 164:255–262.
20. **Mozes, N., and P. G. Rouxhet.** 1987. Methods for measuring hydrophobicity of microorganisms. *J. Microbiol. Methods* 6:99–112.
21. **Nesbitt, W. E., R. J. Doyle, and K. G. Taylor.** 1982. Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite. *Infect. Immun.* 38:637–644.
22. **Oakley, J. D., K. G. Taylor, and R. J. Doyle.** 1985. Trypsin-susceptible cell surface characteristics of *Streptococcus sanguis*. *Can. J. Microbiol.* 31:1103–1107.
23. **Olsson, J., and G. Westergren.** 1982. Hydrophobic surface properties of oral streptococci. *FEMS Microbiol. Lett.* 15:319–323.
24. **Pshigorski, I., J. Gabbay, I. Eli, S. Brenner, R. Bar-Ness, and M. Rosenberg.** 1987. A novel approach to halitosis measurement. *J. Dent. Res.* 66:911.
25. **Rosan, B., R. Wifert, and E. Golub.** 1985. Bacterial surfaces, salivary pellicles, and plaque formation, p. 69–76. *In* S. E. Mergenhagen and B. Rosan (ed.), *Molecular Basis of Oral Microbial Adhesion*. American Society for Microbiology, Washington, D.C.
26. **Rosenberg, E., A. Gottlieb, and M. Rosenberg.** 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. *Infect. Immun.* 39:1024–1028.
27. **Rosenberg, E., N. Kaplan, O. Pines, M. Rosenberg, and D. Gutnick.** 1983. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbons. *FEMS Microbiol. Lett.* 17:157–160.
28. **Rosenberg, M., H. Judes, and E. Weiss.** 1983. Desorption of adherent bacteria from a solid hydrophobic surface by oil. *J. Microbiol. Methods* 1:239–244.
29. **Rosenberg, M., H. Judes, and E. Weiss.** 1983. Cell surface hydrophobicity of dental plaque microorganisms in situ. *Infect. Immun.* 42:831–834.
30. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: role in bacterial adhesion. *Adv. Microb. Ecol.* 9:353–393.
31. **Rosenberg, M., E. Rosenberg, H. Judes, and E. Weiss.** 1983. Bacterial adherence to hydrocarbons and to surfaces in the oral cavity. *FEMS Microbiol. Lett.* 20:1–5.

31a. **Rosenberg, M., E. Weiss, and I. Eli.** 1988. A novel replica technique for localization of caries-associated bacteria on tooth surfaces: development and initial experience. *Caries Res.* 22:42–44.

32. **Sar, N., and E. Rosenberg.** 1987. Fish skin bacteria: colonial and cellular hydrophobicity. *Microb. Ecol.* 13:193–202.
33. **Solis-Gaffar, M. C., H. P. Niles, W. C. Rainieri, and C. Kestenbaum.** 1975. Instrumental evaluation of mouth odor in a human clinical study. *J. Dent. Res.* 54:351–357.
34. **Svanberg, M., G. Westergren, and J. Olsson.** 1984. Oral implantation in humans of *Streptococcus mutans* strains with different degrees of hydrophobicity. *Infect. Immun.* 43:817–821.
35. **Tal, M., E. Weiss, H. Judes, and M. Rosenberg.** 1984. Adherence of dispersed plaque bacteria to buccal epithelial cells. *J. Dent. Res.* 63:547.
36. **Tonzetich, J.** 1977. Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J. Periodontol.* 48:13–20.
37. **van der Mei, H. C., A. H. Weerkamp, and H. J. Busscher.** 1987. A comparison of various methods to determine hydrophobic properties of streptococcal cell surfaces. *J. Microbiol. Methods* 6:277–287.

37a. **van der Mei, H. C., A. H. Weerkamp, and H. J. Busscher.** 1987. Physico-chemical surface characteristics and adhesive properties of *Streptococcus salivarius* strains with defined cell surface structures. *FEMS Microbiol. Lett* 40:15–19.

38. **Weerkamp, A. H., H. C. van der Mei, and J. W. Slot.** 1987. Relationship of cell surface morphology and composition of *Streptococcus salivarius* K+ to adherence and hydrophobicity. *Infect. Immun.* 55:438–445.
39. **Weiss, E., H. Judes, and M. Rosenberg.** 1985. Adherence of a non-oral hydrophobic bacterium to the human tooth surface. *Dent. Med.* 3:11–13.
40. **Weiss, E., M. Rosenberg, H. Judes, and E. Rosenberg.** 1982. Cell surface hydrophobicity of adherent oral bacteria. *Curr. Microbiol.* 7:125–128.
41. **Westergren, G., and J. Olsson.** 1983. Hydrophobicity and adherence of oral streptococci after repeated subculture in vitro. *Infect. Immun.* 40:432–435.
42. **Wilson, P. A. D., W. M. Edgar, and S. A. Leach.** 1984. Some physical properties of oral streptococci that might have a role in their adhesion to oral surfaces, p. 99–112. *In* J. M. ten Cate, S. A. Leach, and J. Arends (ed.), *Bacterial Adhesion and Preventive Dentistry*. IRL Press, Oxford.
43. **Wyatt, J. E., L. M. Hesketh, and P. S. Handley.** 1987. Lack of correlation between fibrils, hydrophobicity and adhesion for strains of *Streptococcus sanguis* biotypes I and II. *Microbios* 50:7–15.

Saliva-mediated inhibition of adhesion of hydrophobic oral and non-oral bacteria to polystyrene

I. ELI[1], N. REGIMOV[1] and M. ROSENBERG[1/2]

INTRODUCTION

There is increasing evidence that most oral bacteria possess hydrophobic surface properties (Rosenberg and Kjelleberg, 1986). Similarly, hydrophobic properties have been reported for various oral surfaces (Glantz, 1977). Such findings have led to the hypothesis that hydrophobic interactions may play a significant role in mediating adhesion within the oral cavity (Rosenberg *et al.*, 1983).

Since hydrophobic interactions are extremely sensitive to the composition of the bulk aqueous medium, saliva may play a larger role in mediating oral adhesion than is generally assumed. The aim of the present investigation was to study how saliva affects bacterial adhesion to a defined hydrophobic surface, namely polystyrene. The results indicate that (i) saliva inhibits adhesion of both oral and non-oral bacteria to polystyrene in a concentration-dependent manner; (ii) the inhibitory components can be extracted from the saliva by hexadecane, and are probably of low molecular weight ($<$ 15 kD).

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[1] School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel 69978.

[2] Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv, Israel 69978.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Streptococcus mutans OMZ 176N and *Serratia marcescens* RZ were employed. Cells were inoculated 1:200 and grown 18 h. with shaking at 30°C in brain heart infusion broth (Difco Laboratories, Detroit, Michigan). Cells were harvested by centrifugation, washed twice and suspended in phosphate buffered saline (PBS; 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and distilled water to 1 liter, pH 7.2) to a corrected optical density of 6-7, as measured in a Uvikon (Kontron, Zurich, Switzerland) spectrophotometer at 540 nm.

Saliva

Unstimulated whole saliva was collected from one of us, 1-2 hours following breakfast, into a centrifuge tube and immediately clarified by centrifugation at 10,000 g for 10 min. Saliva was then diluted in two-fold serial dilutions with PBS and used promptly.

Adhesion assay

To 2 ml of bacterial suspension, 4 ml of saliva, or serial dilution thereof, was added, and the mixture vortexed vigorously for two minutes. Two hundred µl aliquots were added to wells of flat-culture treated polystyrene microtitre plates (Nunc, Roskilde, Denmark) and incubated for 15 minutes at room temperature. Plates were then rinsed thoroughly with running water, and the adhering bacteria stained for 15 min. with Gentian Violet (Rosenberg, 1981; Rosenberg, 1984). Adhesion was measured as the absorbance at 550 nm, using a Titertek Multiscan MC (Flow Laboratories, McLean, Virginia) microtitre plate reader. As a control, 4 ml of PBS was substituted for saliva dilution.

Fractionation of Saliva

For hexadecane extraction, 2 ml of saliva were diluted with 2 ml of PBS, and 2 ml of hexadecane was added. The phases were then vortexed for 2 min. Following phase separation, the lower aqueous phase was carefully removed, hexadecane was again added and the extraction procedure repeated, to ensure complete removal of hexadecane from the lower aqueous phase. This was then removed for adhesion tests. Dialysis of saliva was carried out in an Minicon concentrator B15 (15 kD cutoff) (Amicon Corp., Danvers, MA 09123, USA) at 4° C, and concentrated ten-fold. The saliva was then diluted to its original volume with PBS to test its effect on *S. marcescens* adhesion.

Lysozyme activitiy

In order to assay lysozyme activity, 25 μl aliquots from untreated and fractionated saliva were applied in quadruplicate to paper pads (Schleicher and Schull, West Germany). Following drying, the pads were placed on lawns of *Micrococcus lysodeikticus* (ATCC 4698) which were spread on nutrient agar plates. Following 18 h. incubation, at 30° C, the diameters of the zones of inhibition were compared with those obtained using known salivary dilutions, and the relative activity obtained by extrapolation.

RESULTS

A typical experiment of adhesion of *S. mutans* and *S. marcescens* to polystyrene, as a function of salivary dilution, is shown in Fig. 1. Whole, unextracted saliva inhibited adhesion by 86% and 91%, respectively, as compared to the control containing no saliva. Inhibition decreased with decreasing salivary concentration. Inhibition was apparently due to coating of the polystyrene surface, rather than the cells themselves; pre-incubation of *S. marcescens* cells in saliva, followed by washing and resuspension in PBS did not lower adhesion. However, pre-coating of the polystyrene surface with saliva reduced adhesion by 78% (data not shown).

The inhibitory effect of saliva on the observed adhesion could be abolished by extracting the saliva samples with hexadecane prior to the adhesion assay. Adhesion of *S. marcescens* in the presence of dilutions of extracted compared with non-extracted saliva is shown in Table I. Among the components which were extracted by hexadecane was lysozyme, as evidenced by a 70% decrease in activity in the bulk salivary phase, following the mixing (Fig. 2).

Saliva which was dialyzed in a Minicon B15 lost 81% of its inhibitory ability, as compared to the un-dialyzed control, suggesting that low molecular weight (< 15 kD) components may be responsible for the observed

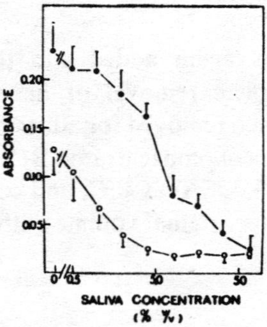

Fig. 1. Effect of saliva on adhesion of *S. mutans* OMZ 176N and *S. marcescens* RZ to polystyrene. The lower curve (○) illustrates the effect of salivary dilutions on adhesion of *S. mutans*, and the upper curve (●), on adhesion of *S. marcescens*. Each point represents the arithmetic mean ± standard deviation.

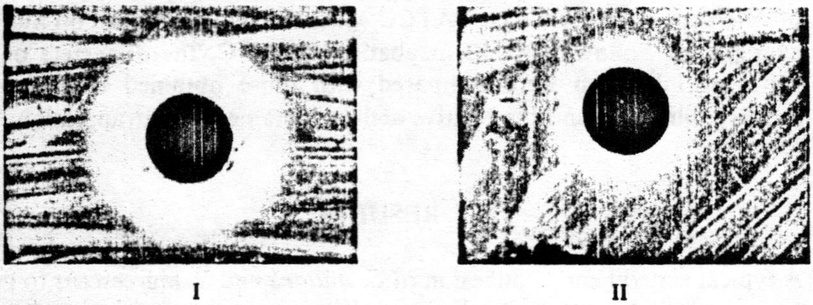

Fig. 2. Effect of hexadecane extraction of saliva on lysozyme activity. Adsorption and desorption of lysozyme from clarified human saliva. Lysozyme activity was assayed on lawns of *M. lysodeikticus* as described in Materials amd Methods. (I) Zone on inhibition of clarified human saliva; (II) Loss of activity following mixing with hexadecane.

TABLE I

Effect of hexadecane extraction of saliva-mediated inhibition of «S. marcescens» adhesion to polystyrene

Salivary concentration (% v/v)	Relative Adhesion[1]	
	Non-extracted Saliva	Extracted Saliva
0	1.00	1.00
0.5	0.96	1.04
3.1	0.86	1.00
13	0.50	1.01
33	0.13	1.05

[1] Relative adhesion to polystyrene, as compared to that of control in the absence of saliva (0.180).

inhibition of adhesion. Of interest was the observation that lysozyme was also among the salivary components lost following the Minicon B15 dialysis procedure (not shown).

DISCUSSION

In most previous investigations, the effect of saliva on adhesion was studied under conditions simulating those in the oral cavity, such as saliva-coated hydroxyapatite (Gibbons, 1980). However, in such systems, the relative contributions of specific (lectin-like) and non-specific (hydrophobic) interactions are difficult to ascertain. In order to focus on the latter, we chose to study the effect of saliva on adhesion to polystyrene. The data demonstrate that component(s) present in saliva inhibit bacterial adhesion to polystyrene in a dose-dependent fashion. The factor(s) which mediate this phenomenon can be extracted from the bulk salivary phase by mixing with hexadecane. The results suggest that the observed inhibition of adhesion is due to the presence of relatively small hydrophobic molecules, capable of interacting with hexadecane, as well as with polystyrene substratum. Whereas loss of lysozyme activity, both following dialysis and hexadecane extraction, was concomitant with loss of inhibitory activity, its possible role in this phenomenon remains to be determined. In addition to low molecular weight proteins, amphipathic polysaccharides and lipid constituents may be involved in the observed inhibition.

Polystyrene is a useful and convenient hydrophobic surface for the study of bacterial adhesion and adhesion-related effects, such as identification of adherent, hydrophobic colonies (Rosenberg, 1981), and desorption of bound cells (Rosenberg, Judes and Weiss, 1983). Several recent studies have employed this substratum to study the adhesion properties of oral strains of bacteria. For example, the lack of ability of a *Streptococcus sanguis* mutant to adhere to polystyrene correlated with its inability to adhere to saliva-coated hydroxyapatite (Gibbons, Etherden and Skobe, 1983). Similarly, adherence of *S. mutans* to microtitre wells has been employed to study complementation of adhesion-deficient mutants (Larrimore *et al.*, 1983). The present study suggests further use of this substratum in the study of the relationship between saliva and bacterial adhesion.

SUMMARY

The effect of saliva on adhesion of oral and non-oral bacteria to a hydrophobic surface (polystyrene) was studied. Washed bacterial suspensions of

Streptococcus mutans OMZ 176N and *Serratia marcescens* RZ were mixed with serial dilutions of clarified human saliva, and the mixtures were incubated in wells of untreated polystyrene microtitre plates. Following washing, to remove unbound cells, the adherent cells were stained with Gentian Violet. Adhesion was measured spectrophotometrically in an ELISA reader at 550 nm. Saliva inhibited adhesion of *S. mutans* OMZ 176N and *S. marcescens* RZ in a concentration-dependent manner, by up to 84% and 91%, respectively. Extraction of saliva with hexadecane, prior to the adhesion assay, brought about an almost complete loss of salivary-mediated inhibition. Preliminary data suggest that the factor(s) mediating the observed inhibition are low (< 15 kD) molecular weight, and may include salivary lysozyme.

REFERENCES

Gibbons, R.J. (1980). In: Adsorption of Microorganisms to Surfaces (Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B., Eds.), pp. 351-388, Ellis Horwood, Chichester, England.

Gibbons, R.J., Etherden, I. and Skobe, Z. (1983). *Infect. Immun.* **41**, 414.

Glantz, P.O. (1977). *Odontologisk Revy* **20** (Supplement 17), 1.

Larrimore, S., Murchison, H., Shiota, T., Michalek, S.M. and Curtis, T., III (1983). *Infect. Immun.* **42**, 558.

Rosenberg, M. and Kjelleberg, S. (1986). *Adv. Microbial. Ecol.* **9**, 353.

Rosenberg, M., Rosenberg, E., Judes, H. and Weiss, E. (1983). FEMS, *Microbiol. Lett.* **20**, 1.

Rosenberg, M. (1981). *Appl. Environ. Microbiol.* **42**, 375.

Rosenberg, M. (1984). *J. Bacteriol.* **160**, 480.

Rosenberg, M., Judes, H. and Weiss, E. (1983). *J. Microbiol. Methods* **1**, 239.

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Putative Role of a 70 kDa Outer-surface Protein in Promoting Cell-surface Hydrophobicity of *Serratia marcescens* RZ

By RONIT BAR-NESS[1,2]* AND MEL ROSENBERG[1,3]

The Maurice and Gabriela Goldschleger School of Dental Medicine[1]*, the Department of Physiology and Pharmacology*[2]*, and the Department of Human Microbiology*[3]*, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel*

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Serratia marcescens RZ has been previously shown to possess pronounced cell-surface hydrophobicity, as evidenced by its affinity for hydrocarbons and polystyrene. The present report suggests the involvement of a 70 kDa protein, serraphobin, in this phenomenon. The 70 kDa protein was recovered from both the cell surface and culture supernatant of hydrophobic wild-type cells, but was either totally absent or present in minor quantities in hydrophobicity-deficient mutants. Similarly, loss of hydrophobicity of RZ cells following growth at 39 °C was accompanied by loss of the protein. Serraphobin was capable of binding to hexadecane droplets following a brief mixing procedure, and could be desorbed by solidifying and melting the hexadecane phase.

INTRODUCTION

Serratia marcescens is a micro-organism of both ecological and medical importance (Yu, 1979; Sleigh, 1983). *Serratia* strains adhere avidly to a variety of hydrophobic surfaces such as air/water and oil/water interfaces (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1982; Kefford *et al.*, 1982; Syzdek, 1982) and to various non-wettable plastics (Rosenberg, 1981). Hydrophobicity of *Serratia* has been suggested as a survival mechanism in the open environment (Kefford *et al.*, 1982). Similarly, in a recent study of the adhesion properties of 14 clinical *S. marcescens* isolates, all were capable of adhering to hydrocarbons and polystyrene, suggesting a possible role for cell-surface hydrophobicity in initial host colonization (Rosenberg *et al.*, 1986*a*).

Previous investigations have reported correlations between hydrophobicity in *Serratia* and production of the pigment prodigiosin (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1979, 1982; Kefford *et al.*, 1982; Syzdek, 1982). However, non-pigmented clinical strains (Rosenberg *et al.*, 1986*a*) and mutants (Rosenberg, 1984) may also exhibit intermediate or pronounced hydrophobic surface properties, suggesting the participation of additional factor(s).

In the present study, correlations between hydrophobicity of *S. marcescens* RZ and the presence and level of a major 70 kDa surface protein (which we have named serraphobin), are reported.

METHODS

Bacterial strains and growth conditions. S. marcescens strain RZ was originally obtained from R. Zack, Tel Aviv University. Mutant 3162, exhibiting intermediate cell-surface hydrophobicity, and mutant 3164, almost completely devoid of cell-surface hydrophobicity were derived from strain RZ as previously reported (Rosenberg, 1984). The bacteria were maintained on brain heart infusion (BHI) agar (Difco) at 4 °C and transferred every month. Starter cultures were prepared by inoculating bacteria into tubes containing 2 ml PG broth [5 g Bactopeptone, (Difco); 10 ml glycerol, distilled water to one litre] or BHI broth. The tubes were incubated for 24 h with shaking at 30 or 39 °C. Bacteria were then inoculated into flasks containing 20 ml of PG or BHI broth to an

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initial cell concentration of 5×10^5 c.f.u. ml^{-1}. Following 72 h incubation at 30 or 39 °C in a G76 gyratory shaker (New Brunswick Scientific) at 300 r.p.m., samples were taken for c.f.u. determination. Bacteria were harvested by centrifugation at 10000 ***g***, and wet weights were determined.

Spent culture medium. The culture supernatants were sterilized by filtration and stored at −20 °C for hexadecane extraction and SDS-PAGE.

Cell pellet extractions. The cell pellets were extracted by 2% (w/v) SDS/5% (v/v) β-mercaptoethanol as described by McBride *et al.* (1985). Pellets were washed twice in 0·15 M-NaCl by centrifugation for 20 min at 10000 ***g***. Pellets were weighed and subsequently suspended 1:1 (w/v) in 2% SDS/5% β-mercaptoethanol, vigorously agitated on a vortex mixer and boiled for 30 min. The mixtures were then centrifuged for 10 min at 6000 ***g***. The supernatants were stored at −20 °C for SDS-PAGE.

SDS-PAGE. This was performed according to the method of Laemmli (1970). Samples of spent culture medium were diluted in water, correcting for differences in wet weights of cell pellets following growth. Samples of outer-surface protein extractions were diluted either 1:4 or 1:8 in distilled water. Sample buffer (1 vol.) containing 40 mmol l^{-1} dithiothreitol (DTT) was added to 3 vols of sample, and the mixture was boiled for 2 min. Samples (40 μl) and standards (12 μl; molecular mass markers, 14·2–66 kDa, Sigma) were layered on to 7·5% or 10% polyacrylamide gels, as indicated. Following electrophoresis at 200 V, protein bands were visualized by staining with Coomassie blue. Since the presence of pigment precluded protein measurement by standard spectrophotometric techniques, the total amounts of protein in each sample were estimated by densitometric comparisons of each lane with that of the molecular mass marker proteins, using a CAMAG TLC Scanner II (Camag, Switzerland); the percentage of serraphobin with respect to total protein was similarly estimated densitometrically.

Hexadecane extraction. Hexadecane extraction of spent culture fluids was carried out essentially as previously described (Rosenberg *et al.*, 1986*b*). Hexadecane (0·5 ml) was added to 0·5 ml culture supernatant supplemented with 0·5 ml phosphate buffer (44·4 g K_2HPO_4, 14·52 g KH_2PO_4, distilled water to one litre, pH 7·1). The aqueous hydrocarbon mixtures were then vortexed for 2 min on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuqe, Iowa, USA) at room temperature. Following phase separation, the lower aqueous phase was removed carefully using a Pasteur pipette to another test tube containing an additional 0·5 ml hexadecane, and the procedure was repeated. Following four such extraction cycles the final lower aqueous phase was stored at −20 °C for SDS-PAGE. Half-strength phosphate buffer (0·25 ml) was added to the hexadecane portions. The mixtures were cooled to 0 °C until solidification of the hexadecane was complete. The test tubes were then incubated at 30 °C until the hexadecane melted. The test tubes were cooled and heated in this manner three consecutive times; the final mixture was stored at −20 °C for at least 18 h. Prior to SDS-PAGE, the test tubes were allowed to reach room temperature, and samples for SDS-PAGE were removed from the lower aqueous phase.

Cell-surface hydrophobicity. Bacterial hydrophobicity was determined by adhesion to hexadecane (Rosenberg, 1981, 1984). To 1·4 ml bacterial suspension, washed and suspended in half-strength phosphate buffer, 0·2 ml hexadecane was added and the mixtures vortexed in semi-micro polystyrene disposable cuvettes for 60 s. Following phase separation, the absorbance of the lower aqueous phase at 400 nm was determined. Adhesion is expressed as the percentage decrease in turbidity of the lower aqueous phase following mixing, compared to that of the initial cell suspension (Rosenberg, 1984).

RESULTS

Hydrophobic *Serratia marcescens* RZ cells, grown at 30 °C on PG or BHI medium, produced an extracellular protein (serraphobin) with a molecular mass of approximately 70 kDa, which was one of the major proteins (25% of total supernatant protein) recovered from the culture supernatant (Fig. 1, lane 1). Serraphobin appeared to be totally absent in spent culture media of non-hydrophobic mutant 3164 (Fig. 1, lane 2) and non-pigmented mutant 3162 (Fig. 1, lane 3). A second supernatant protein of about 50 kDa was present in wild-type RZ cells and mutant 3164 cells (Fig. 1, lanes 1, 2), but was absent from spent culture media of mutant 3162 (Fig. 1, lane 3). The absence of this protein may be related to loss of pigmentation in mutant 3162.

In order to determine whether serraphobin is located on the outer surface of RZ cells, treatment with 2% SDS/5% β-mercaptoethanol, as described by McBride *et al.* (1985), was employed (Fig. 1, lanes 4–6). Serraphobin was extracted in relatively large amounts (5% of total protein, lane 4) from wild-type RZ cells, as opposed to no appreciable amounts in non-hydrophobic 3164 cells (lane 5), and minimal quantities (0·4% of total protein) in partially hydrophobic 3162 cells (lane 6). Serraphobin was also extracted from wild-type RZ cells following 1% SDS treatment (data not shown), as described by Jenkinson (1986). Thus, this protein appears to be a cell-surface component of the outer surface of RZ.

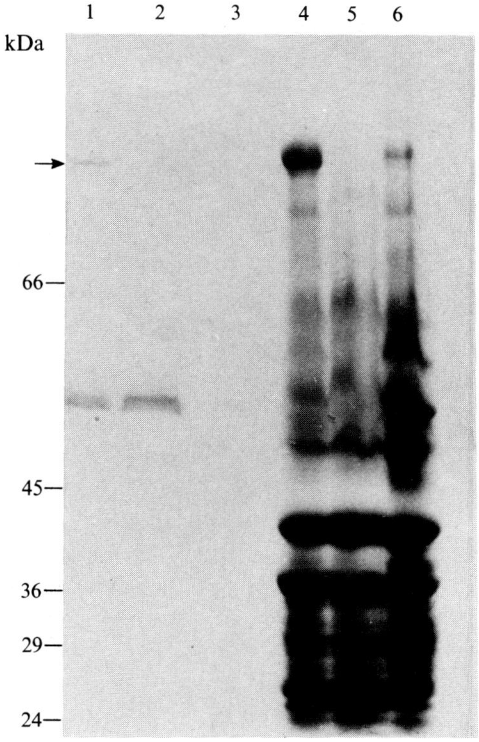

Fig. 1. SDS-PAGE of culture supernatants and outer-surface protein extracts following growth at 30 °C in BHI broth. Culture supernatant samples were diluted with water to correct the differences in wet weights of pellets following growth: RZ, 46·9 mg ml^{-1}; mutant 3164, 29·1 mg ml^{-1}; mutant 3162, 40·6 mg ml^{-1}. About 2 μg of protein of each sample was loaded onto a 7·5% polyacrylamide gel. Outer-surface proteins were extracted from washed cell suspensions, as described in Methods. Samples were diluted 2·5-fold in water. Approximately 250 μg of protein of each sample was loaded onto the gel. Protein bands were visualized by Coomassie blue staining. Adhesion to hexadecane was 76% for RZ cells, 0% for 3164 cells, and 28% for strain 3162. Lanes: culture supernatants of RZ (lane 1), 3164 (lane 2) and 3162 (lane 3); outer surface protein extracts of RZ (lane 4), 3164 (lane 5) and 3162 (lane 6). Serraphobin is indicated by the arrow. Molecular mass standards: bovine serum albumin (66 kDa); ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20 kDa).

Serraphobin, released from the cell surface, exhibited affinity for hexadecane droplets (Fig. 2). Mixing with hexadecane removed 92% of the protein from the aqueous phase (lane 2). Its partitioning to the oil/water interface could be demonstrated by subsequent desorption (lane 3) following solidification of the hexadecane droplets at below 18 °C, a technique recently proposed for separation of amphipathic proteins (Rosenberg *et al.*, 1986*b*). Another major culture supernatant protein of about 42 kDa was also eluted by hexadecane. This 42 kDa protein was also present in the non-hydrophobic mutant 3164, and remained following growth at 39 °C.

Previous studies have shown that cell-surface hydrophobicity in strain RZ and other *S. marcescens* isolates is lost when the cells are grown at temperatures above 38 °C (Rosenberg *et al.*, 1986*a*). Fig. 3 compares the proteins extracted from the cell surfaces of RZ, 3162 and 3164 grown at 39 °C. Serraphobin was found neither in cell-surface extracts (Fig. 3, lanes 2–4), nor in the spent culture medium (not shown) of any of the strains.

DISCUSSION

The present study suggests the involvement of a 70 kDa surface protein, serraphobin, in mediating cell-surface hydrophobicity in *S. marcescens* RZ. Serraphobin was recovered from

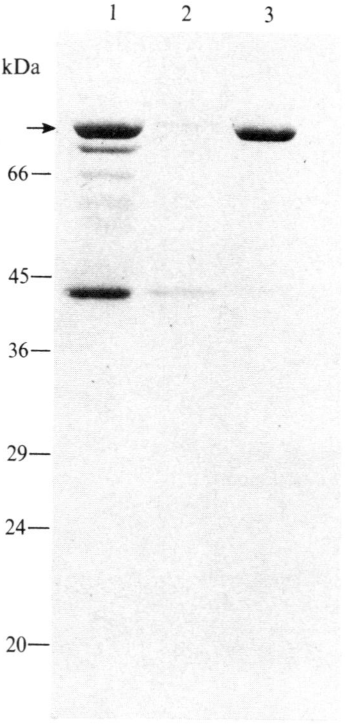

Fig. 2

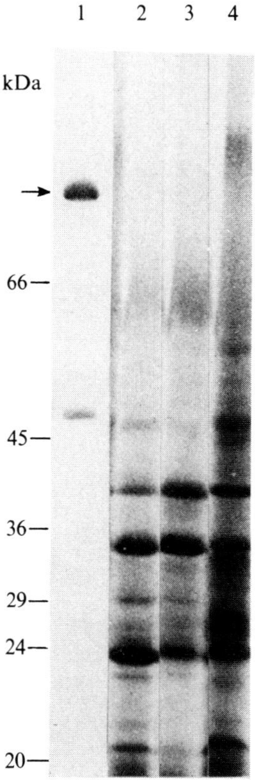

Fig. 3

Fig. 2. SDS-PAGE of culture supernatant of *S. marcescens* RZ following growth at 30 °C on PG medium, before and after hexadecane extraction and desorption. Samples were applied onto a 10% gel. Protein bands were visualized by Coomassie blue staining. Adhesion of washed cells to hexadecane was 71%. Lanes: 1, RZ culture supernatant prior to hexadecane extraction (about 10 μg protein was loaded onto the gel); 2, RZ culture supernatant following 8 min mixing with hexadecane; 3, desorption following solidification and melting of the upper phase following mixing with hexadecane. Twenty-five percent of the adsorbed serraphobin was recovered by the desorption procedure, as determined by densitometry. Serraphobin is indicated by the arrow. Molecular mass standards, see Fig. 1.

Fig. 3. SDS-PAGE of cell-surface extracts following growth at 39 °C. Samples of cell-surface extracts following growth at 39 °C (approx. 20 μg protein per sample) were applied to a 7·5% gel: lane 2, RZ; lane 3, 3164; lane 4, 3162. As control, culture supernatant of RZ cells grown at 30 °C (2 μg protein) was applied to lane 1. Protein bands were visualized by Coomassie blue staining. Adhesion to hexadecane was less than 10% for all strains. Serraphobin is indicated by the arrow. Molecular mass standards, see Fig. 1.

both the cell surface and culture supernatant of hydrophobic wild-type cells, but was absent in non-hydrophobic mutant 3164, and present in minor quantities on the surface of partially hydrophobic 3162 mutant cells. Similarly, loss of hydrophobicity of RZ cells following growth at 39 °C was accompanied by loss of serraphobin. Serraphobin was capable of binding to the hexadecane/water interface following a brief mixing procedure, and could be desorbed by solidifying the hexadecane phase. Preliminary data (not shown) show a correlation between serraphobin production and cell-surface hydrophobicity in several clinical *S. marcescens* isolates.

Recent studies have shown that cell-surface hydrophobicity in *Serratia* strains is more complicated than previously anticipated, involving an interplay of various 'hydrophobins' (hydrophobicity-promoting factors) and 'hydrophilins' (hydrophobicity-reducing components). For example, whereas earlier investigations of hydrophobicity in *S. marcescens* suggest that prodigiosin is chiefly responsible for this phenomenon, more recent studies have demonstrated that non-pigmented isolates and mutants can also exhibit hydrophobic properties (Blanchard & Syzdek, 1978; Hermansson *et al.*, 1979, 1982; Rosenberg, 1981, 1984), suggesting that additional hydrophobins are involved. Based on the present data, serraphobin appears to be a hydrophobin in RZ cells. Conversely, the potential role of cell-surface components which reduce hydrophobicity must also be considered. For example, serratamolide, an amphipathic cell-surface component of some *S. marcescens* strains (including RZ) acts as a hydrophilin (Bar-Ness *et al.*, 1988).

Investigations are presently under way to further characterize serraphobin, its distribution in clinical *Serratia* strains, and its role in promoting adhesion of *S. marcescens* to hydrophobic surfaces.

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REFERENCES

Bar-Ness, R., Avranamy, N., Matsuyama, T. & Rosenberg, M. (1988). Increased cell surface hydrophobicity of a *Serratia marcescens* NS 38 mutant lacking wetting activity. *Journal of Bacteriology* **170**, 4361–4364.

Blanchard, D. C. & Syzdek, L. D. (1978). Seven problems in bubble and jet drop researches. *Limnology and Oceanography* **23**, 389–400.

Hermansson, M., Kjelleberg, S. & Norkrans, B. (1979). Interaction of pigmented wild type and pigmentless mutant of *Serratia marcescens* with lipid surface film. *FEMS Microbiology Letters* **6**, 129–132.

Hermansson, M., Kjelleberg, S., Korhonen, T. K. & Stenstrom, T. A. (1982). Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion to an air–water interface. *Archives of Microbiology* **131**, 308–312.

Jenkinson, H. F. (1986). Cell-surface proteins of *Streptococcus sanguis* associated with cell hydrophobicity and coaggregation properties. *Journal of General Microbiology* **132**, 1575–1589.

Kefford, B., Kjelleberg, S. & Marshall, K. C. (1982). Bacterial scavenging: utilization of fatty acids localized at a solid–liquid interface. *Archives of Microbiology* **133**, 257–260.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

McBride, B. C., Morris, E. J. & Ganeshkumar, N. (1985). Relationship of streptococcal cell surface proteins to hydrophobicity and adherence. In *Molecular Basis of Oral Microbial Adhesion*, pp. 85–93. Edited by S. Mergenhagen & B. Rosan. Washington, DC: American Society for Microbiology.

Rosenberg, M. (1981). Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Applied and Environmental Microbiology* **42**, 375–377.

Rosenberg, M. (1984). Isolation of pigmented and non-pigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. *Journal of Bacteriology* **160**, 480–482.

Rosenberg, M., Blumberger, Y., Judes, H., Bar-Ness, R., Rubinstein, E. & Mazor, Y. (1986*a*). Cell surface hydrophobicity of pigmented and non-pigmented clinical *Serratia marcescens* strains. *Infection and Immunity* **51**, 932–935.

Rosenberg, M., Zosim, Z., Regimov, N. & Eli, I. (1986*b*). Separation of amphipathic proteins based on adsorption to hexadecane: water interfaces. *Preparative Biochemistry* **16**, 133–141.

Sleigh, J. D. (1983). Antibiotic resistance in *Serratia marcescens*. *British Medical Journal* **287**, 1651–1653.

Syzdek, L. D. (1982). Concentration of *Serratia* in the surface microlayer. *Limnology and Oceanography* **26**, 961–964

Yu, V. L. (1979). *Serratia marcescens* – historical perspective and clinical review. *New England Journal of Medicine* **300**, 887–891.